PROGRAMMING OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS DURING FETAL LIFE

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DECLARATION

I declare that this thesis was written by me and that the data presented within it is the result of my own work, except for the assays below, and where otherwise acknowledged in the text or on the following page.

Cortisol, dehydropiandrosterone and 17-α-hydroxyprogesterone were measured in plasma and creatinine was measured in urine by Dr P Wood in the Regional Endocrine Unit, Southampton General Hospital, Southampton, SO16 6YD.

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This work has not been submitted for any other degree.

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Abstract

In epidemiological studies low birthweight predicts development of hypertension, insulin resistance, glucose intolerance and dyslipidaemia (together described as the 'Metabolic Syndrome') and subsequent cardiovascular disease in adult life. The mechanisms are unknown but may involve disturbances of the hypothalamic-pituitary-adrenal (HPA) axis.

In animal models, manipulation of the *in utero* environment by maternal undernutrition, treatment with dexamethasone or physical restraint, results in low birthweight offspring with hypertension and insulin resistance in adult life. These early life events have long-term effects on the HPA axis, resulting in elevated basal and stress-induced glucocorticoid secretion and subtle changes in HPA axis regulation. Glucocorticoid receptor (GR) and mineralocorticoid receptor expression are decreased in the hippocampus, one of the most sensitive targets for glucocorticoid negative feedback effects in rodents, but increased in the amygdala, a site of positive modulation of HPA axis activity.

Although the human evidence is limited, glucocorticoid excess (in Cushing's syndrome) is associated with development of the Metabolic Syndrome and therefore it is plausible that more subtle variations in glucocorticoid action contribute to the pathogenesis of risk factors for cardiovascular disease. Moreover, in adult men, higher fasting morning plasma cortisol concentrations are associated with higher blood pressure, plasma glucose and triglyceride concentrations, and lower birthweight. These studies have led to the hypothesis that events in early life permanently alter or 'programme' cortisol secretion and that this leads to a high prevalence of the Metabolic Syndrome and cardiovascular disease in adulthood. The aim of this thesis was to study the HPA axis in detail, examining aspects of both activity of the axis and cortisol action, in order to determine variations that could explain the link between low birthweight and subsequent development of cardiovascular risk factors.

The principal findings were that men aged 66-77 years, who were of low birthweight and / or with the Metabolic Syndrome, have activation of the HPA axis with increased cortisol response to $ACTH_{1-24}$ and increased urinary cortisol metabolite excretion. In contrast to rats programmed by dexamethasone administration during pregnancy, there was no evidence of altered central feedback sensitivity to low dose dexamethasone. However, dexamethasone may not cross the blood-brain barrier at low doses in man, so that this only tests the pituitary component of the negative feedback loop. Alternatively, rather than impaired central negative feedback, the activation of the HPA axis could be due to increased forward drive to ACTH and cortisol secretion from higher centres. Consistent with this hypothesis there was lack of habituation to the stress of repeated venepuncture in diabetic subjects.

In contrast to the central GR changes, peripheral (liver) GR expression is increased in the rodent model. Tissue-specific differences in GR levels are a potential mechanism whereby subtle abnormalities in cortisol action could contribute to variations in insulin sensitivity in the population. Using competitive quantitative RT-PCR, GR mRNA levels were determined in total RNA extracted from skeletal muscle biopsies from 23 men in Uppsala, Sweden who had been studied at age 70 years with an oral glucose tolerance test and a euglycaemic hyperinsulinaemic clamp. Increased GR expression was associated with resistance to insulin-mediated glucose uptake, independent of obesity. Using 5'-RACE-PCR (Rapid Amplification of cDNA ends) it was demonstrated that one mechanism for tissue-specific differences in GR is effects upon one or more multiple alternate promoters of the GR gene.

Thus variations in HPA axis activity may be a key mechanism to explain the effects of early life events on later cardiovascular disease and offers potential for new therapeutic strategies to reduce cardiovascular risk. Understanding of the molecular mechanisms involved in glucocorticoid programming and the consequences of permanently altered glucocorticoid activity may provide novel insights into the pathogenesis of cardiovascular and metabolic disease.

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Abbreviations

$ACTH_{1-24}$	adrenocorticotrophic hormone
AUC	area under the curve
AVP	arginine vasopressin
BMI	body mass index
bp	base pair
11 β-HSD1	11 beta hydroxysteroid dehydrogenase type 1
11 β-HSD2	11 beta hydroxysteroid dehydrogenase type 2
CBG	corticosteroid binding globulin
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CIP	calf intestinal phosphatase
CRH	corticotrophin releasing hormone
CV	coefficient of variation
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DST	dexamethasone suppression test
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
g	gram
GR	glucocorticoid receptor
h	hour
HDL	high density lipoprotein
HPA	hypothalamic-pituitary-adrenal
IGF	insulin-like growth factor
IGT	impaired glucose tolerance
kb	kilobase
kg	kilogram
l	litre
lb	pound
LDL	low density lipoprotein

M	molar
μg	microgram
μl	microlitre
μM	micromolar
mg	milligram
ml	millilitre
mM	millimolar
min	minute
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
ng	nanogram
nM	nanomolar
OGTT	oral glucose tolerance test
PCR	polymerase chain reaction
PEP-CK	phosphoenolpyruvate carboxykinase
PI	ponderal index
pmol	picomolar
5'-RACE	Rapid Amplification of cDNA Ends
RIA	radioimmunoassay
RLM-RACE	RNA Ligase Mediated Rapid Amplification of cDNA Ends
RNA	ribonucleic acid
RT	reverse transcription
s	second
sd	standard deviation
TAP	tobacco acid phosphatase
TdT	terminal deoxynucleotidyl transferase
THE	tetrahydrocortisone
THF	tetrahydrocortisol
5α-THF	5 alpha tetrahydrocortisol
5β-THF	5 beta tetrahydrocortisol
TRIS	tris (hydroxymethyl) methylamine
WHR	waist-hip-ratio

Publications from this thesis

Papers

Reynolds RM, Bendall HE, Whorwood CB, Wood PJ, Walker BR, Phillips DIW Reproducibility of the low dose dexamethasone suppression test: comparison between direct plasma and salivary cortisol assays. *Clin Endocrinol* 1998; 49: 307-310

Reynolds RM, Walker BR, Syddall HE, Andrew R, Wood PJ, Whorwood CB, Phillips DI Altered control of cortisol secretion in adult men with low birthweight and cardiovascular risk factors *J Clin Endocrinol Metab* 2001; 86: 245-250

Reynolds RM, Walker BR, Syddall HE, Wood PJ, Phillips DI Elevated plasma cortisol in glucose intolerant men: differences in responses to glucose and habituation to venepuncture *J Clin Endocrinol Metab* 2001; 86: 1149-1153

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Chapter 1
Introduction

1.1 Background

Recent epidemiological studies have shown that events in early life are associated with the development of subsequent cardiovascular disease and metabolic disorders. It has been proposed that these associations arise as a result of the phenomenon of 'programming', whereby stimuli during crucial periods of development produce permanent changes in cellular or tissue structure and function (Barker 1998). Although the mechanisms are unknown, several hypotheses have been put forward to explain the epidemiological associations. These include inadequate fetal nutrient supply sufficient to reduce fetal growth (Hales and Barker 1992), certain genetic factors (Dunger *et al.* 1998; Hattersley and Tooke 1999) and programming of major endocrine axes controlling growth and development, particularly the hypothalamic-pituitary-adrenal (HPA) axis (Edwards *et al.* 1993; Seckl 1998). There is growing evidence from animal studies that adverse influences in prenatal or early post-natal life permanently alter the biological and behavioural responses in the adult offspring through long-term changes in the set-point of central and peripheral sensitivity to glucocorticoid hormones. In humans, glucocorticoid excess in Cushing's syndrome is associated with development of higher blood pressure, plasma glucose and triglyceride concentrations (i.e. the Metabolic Syndrome), and therefore more subtle variations in activity of glucocorticoids have been implicated in development of these cardiovascular risk factors. Indeed, higher fasting morning plasma cortisol concentrations are associated with the Metabolic Syndrome and also with lower birthweight (Phillips *et al.* 1998; Phillips *et al.* 2000). These preliminary studies suggest that events in early life permanently alter or 'programme' cortisol secretion and that this may be a fundamental mechanism underlying the association between low birthweight and the development of metabolic disorders and cardiovascular disease in adult life. The aims of this thesis were to determine the aspects of the HPA axis and cortisol action which vary and the consequences of permanently altered glucocorticoid activity, in humans of low birthweight and / or with the Metabolic Syndrome.

1.2 Early life events and disease in adulthood

1.2.1 The fetal origins hypothesis

An increasing number of epidemiological studies have shown events in early life are associated with development of disease in adulthood. In population studies, low birthweight or thinness at birth predicts subsequent Type 2 diabetes mellitus, insulin resistance, hypertension, dyslipidaemia and ischaemic heart disease (Barker *et al.* 1993a; Barker *et al.* 1993b; Fall *et al.* 1995; Curhan *et al.* 1996; Forrester *et al.* 1996; Leon *et al.* 1996; Barker 1998). This has led to the 'fetal origins' hypothesis which proposes that adaptations made by the fetus in response to undernutrition lead to permanent changes in physiology and metabolism which in turn predispose to cardiovascular, metabolic and endocrine diseases in adult life. Environmental factors acting at critical periods of development during which cells are rapidly dividing can 'programme' development of fetal tissues and organ structure and may alter expression of the fetal genome. This leads to permanent effects on a wide range of physiological processes, ultimately resulting in later dysfunction and disease (Barker 1998; Edwards *et al.* 1993).

1.2.1a Coronary heart disease

The proposal that the *in utero* environment could influence subsequent coronary heart disease originated from observations of infant mortality rates among babies born in Britain during the early 1900s. Neonatal death rates differed considerably across the country, being highest in some of the northern industrial towns and the poorer rural areas in the north and west. This geographical pattern closely resembles the variations in death rates in adults from coronary heart disease, suggesting that similar factors contribute to both newborn deaths and adult death rates from coronary artery disease. Thus it was proposed that low rates of growth before birth are linked to the development of coronary heart disease in adult life (Barker and Osmond 1986).

The early epidemiological studies linking the early environment to the aetiology of coronary heart disease were based on studies of men and women in middle and late life whose birthweight and body measurements at birth were routinely recorded. At the turn of the 20th century, when perinatal mortality rates were high and there was

concern about the physical deterioration of the British population, nurses were trained to attend women in childbirth and to advise mothers on how to keep their babies healthy. From 1911, midwives attended all pregnant women in Hertfordshire, U.K., recording each baby's birthweight and notifying the births to the County Medical Officer of Health. The local health visitor visited each baby at home at intervals throughout infancy, recording its illnesses and development on a card. When the baby was one year old and the visits ceased, the card was given to the county health visitor and the details transferred to ledgers. In 1986, the MRC Environmental Epidemiology Unit in Southampton discovered the East Hertfordshire ledgers (which had been maintained until 1945) in the County Record Office. These records were the largest set discovered in a systematic search for archives and records in Britain.

The National Health Service Central Register at Southport was used to trace men and women with intact birth records, who were born in Hertfordshire from 1911 to 1930. Among 10 141 of these men, death rates from coronary heart disease fell progressively with increasing birthweight and weight at 1 year of age (Barker *et al.* 1989). An inverse relationship between low birthweight and coronary artery disease has subsequently been confirmed in studies of men in Uppsala, Sweden (Leon *et al.* 1996); Caerphilly, South Wales (Frankel *et al.* 1996); and Helsinki, Finland (Forsen *et al.* 1997); among 80 000 women in the American nurses study (Rich-Edwards *et al.* 1997); and in men and women in Mysore, South India (Stein *et al.* 1996). These relationships are strong and continuous, and represent birthweights within the normal range, not just severely undersized babies (Barker *et al.* 1990; Curhan *et al.* 1996).

1.2.1b Glucose intolerance, hypertension and the Metabolic Syndrome

The trends in the association of cardiovascular disease and birth size are paralleled by similar associations in two of the major risk factors for cardiovascular disease, Type 2 diabetes mellitus and hypertension. The trends are strong: in 370 Hertfordshire men, aged 65 years, the prevalence of Type 2 diabetes mellitus and impaired glucose tolerance (IGT) fell from 40% among men who weighed $\leq$ 5.5 lb (2.54 kg), to 14% among those who weighed $\geq$ 9.5 lb (4.31 kg) (Hales *et al.* 1991). The trends are also

consistent: the inverse relationship between birth size and altered glucose metabolism has been demonstrated in 30 studies of adults throughout the world (Phillips *et al.* 1998; Law *et al.* 2001), while 70 studies, involving more than 440,000 individuals have replicated the association between birth size parameters and hypertension (Law and Shiell 1996; Huxley *et al.* 2000). Although it is not known at what age the increased risk of disease appears, children of low birthweight have abnormalities in glucose and insulin homeostasis at 4 and 7 years of age (Law *et al.* 1995; Yajnik *et al.* 1995), and have higher blood pressure than their contemporaries at 8 years of age (Moore *et al.* 1996).

Birthweight is a summary measure of fetal growth and development *in utero*. Where data are available on other anthropometric measurements at birth, including body length and head circumference, they show that suboptimal fetal growth, in particular thinness at birth (that is a low ponderal index: birthweight/length^3) or stunting of growth, predicts atherosclerotic vascular disease in later life (Hales *et al.* 1991; Phillips *et al.* 1994a; Lithell *et al.* 1996; Forsen *et al.* 1997). Subjects with thinness at birth have a higher prevalence of the 'Metabolic Syndrome' ('Insulin Resistance Syndrome' or 'Syndrome X'), the coexistence of glucose intolerance, hypertension and dyslipidaemia (Reaven and Hoffman 1987; Barker *et al.* 1993b), suggesting that these diseases result from a common intrauterine insult.

1.2.1c Potential confounding factors

It has been suggested that people who were exposed to an adverse environment *in utero* and failed to grow, continue to be exposed to an adverse environment in childhood and adult life, and it is this later adverse environment that produces the effects attributed to programming *in utero*. But there is little evidence to support this argument. Rather, associations between birthweight and raised adult blood pressure or glucose intolerance, are found in each social group, and are independent of adult lifestyle influences such as smoking, alcohol intake and obesity (Barker *et al.* 1995). Adult obesity does, however, add to the intrauterine effects such that the highest prevalence of Type 2 diabetes mellitus and IGT is seen in those people whom were small at birth but become obese as adults (Hales *et al.* 1991; Lithell *et al.* 1996).

1.3 Mechanisms linking birthweight to later disease

Despite the compelling epidemiological evidence, the molecular and cellular mechanisms that explain a link between reduced fetal growth and later adult disease are not understood.

1.3.1 Genes versus environment

It is conceivable that the link between low birthweight and disease in adulthood reflects the effects of genes that reduce fetal growth and predispose the individual to disease later in life. Insulin, for example, plays a key role in fetal growth and thus a genetically determined impairment of insulin action could explain the association between low birthweight and later diabetes (Hattersley and Tooke 1999). Diabetes caused by a mutation of the glucokinase gene, encoding a glucose-sensing protein expressed in the pancreas regulating insulin secretion, is associated with a 500 g birthweight reduction (Hattersley *et al.* 1998). As well as genes modulating insulin secretion, genes determining insulin resistance could also impair fetal growth, as has been demonstrated in transgenic mice lacking key intermediates in the insulin receptor signalling mechanism (Tamemoto *et al.* 1994). Common allelic variation at the variable number of tandem repeat (VNTR) locus in the insulin promoter region of the insulin gene, a polymorphism previously shown to be associated with insulin resistance and Type 2 diabetes, is linked with a 200 g increase in birthweight (Dunger *et al.* 1998). However, the influence of the polymorphism is much smaller that the stronger birth size effect in the Hertfordshire cohort (Ong *et al.* 1999) and so cannot explain all of the link between low birthweight and Type 2 diabetes.

Several sources of evidence suggest that it is the maternal environment, rather than genetic factors, that is the dominant influence on birth size. The strong influence of the mother is demonstrated in half-sibling studies showing a stronger correlation between the birthweights of half-siblings sharing the same mother, than among those sharing the same father (Morton 1955). The importance of the maternal environment is also supported by animal cross breeding experiments and by embryo transfer experiments; a fetus transferred to a larger uterus will attain a larger birth size (Snow 1989). In monozygotic twin studies, the twin who was lighter at birth is at the

greatest risk of Type 2 diabetes mellitus (Poulsen *et al.* 1997). As these twins have the same genetic background the difference in birthweight must be independent of genetic factors. Further evidence against a genetic basis for the association between low birthweight and adult disease is that prominent post-natal catch-up growth is itself a risk factor for subsequent development of hypertension (Levine *et al.* 1994, Eriksson *et al.* 1999), ischaemic heart disease (Osmond *et al.* 1993) and insulin resistance (Leon *et al.* 1996), suggesting that smallness at birth due to intrauterine environmental growth restraint, rather than genetic smallness, is important in determining the risk of later disease. Moreover genetic factors play a minor role in determining birth weight with up to 62% of the variation in birthweight between individuals estimated as the result of the intrauterine environment (Penrose 1954).

1.3.2 Maternal undernutrition

In animal studies, poor maternal nutrition during crucial periods of fetal growth impairs fetal growth and permanently affects the structure and physiology of several organs and tissues. In rats, dietary restriction of protein during pregnancy lowers birthweight and leads to subsequent glucose intolerance and hypertension in adulthood (Langley and Jackson 1994; Hales *et al.* 1996). A reduced nutrient supply to the fetal guinea pig induced by unilateral uterine artery ligation causes a lifelong increase in blood pressure in the offspring (Persson and Jansson 1992). Thus in the 'thrifty phenotype hypothesis' (Hales and Barker 1992), the undernourished fetus makes metabolic adaptations from which it benefits in the short term by increasing fuel availability. These changes are permanently programmed and become counterproductive in post-natal life when nutrition is not restricted.

The human evidence for the influence of maternal nutrition on the fetal origins of adult disease is less clear. The Dutch famine from 1944 to 1945 was a natural 'experiment' of fetal undernutrition with a defined duration of exposure to famine. Babies born to women starved during the famine in the last trimester of pregnancy were of lower birthweight and developed glucose intolerance in adulthood (Ravelli *et al.* 1998). This effect was independent of obesity. However, a cross-sectional study of people born during and after the extreme siege of Leningrad in World War II

showed no relationship between maternal undernutrition and subsequent diabetes mellitus or hypertension (Stanner *et al.* 1997). Thus, maternal malnutrition seems unlikely to be solely responsible for explaining the associations between birth weight and subsequent adult disease. Moreover the importance of variations in maternal nutrition within the 'normal range' as opposed to extreme starvation needs to be established.

1.3.3 Glucocorticoids

An alternative mechanism by which reduced fetal growth could cause long term effects is by either altering the set-point of hormonal axes that control growth and development, or by changing the threshold of response of the tissue to the hormonal stimulus. In response to undernutrition or other prenatal stressful stimuli, the fetus reduces insulin secretion and increases secretion of several hormones that modulate fetal and placental metabolism to increase fuel availability, redistribute blood flow, and alter the rate and pattern of fetal growth. Levels of catabolic hormones such as cortisol, catecholamines and β-endorphins are increased, while concentrations of anabolic hormones or growth factors including thyroxine, insulin, insulin-like growth factors (IGFs) fall (Owens *et al.* 1989). Many of these hormones have well characterised programming or imprinting effects, which occur during specific developmental windows and persist throughout life. Recent studies have suggested that the HPA axis plays a particularly important role and it has been proposed that fetal exposure to glucocorticoids may link low birthweight and the predisposition to diseases in adulthood (Edwards *et al.* 1993; Seckl 1998) (Figure 1.1). This hypothesis is based on several pieces of evidence.

1.3.3a Steroids and programming

Steroid hormones have well-characterised programming effects. Neonatal androgen secretion permanently programmes metabolic pathways in the liver, sexual differentiation of the hypothalamus, and sexual behaviour, effects that persist irrespective of subsequent sex steroid manipulations (Arai and Gorski 1968). Similarly, neonatal stress permanently programmes the pattern of HPA axis

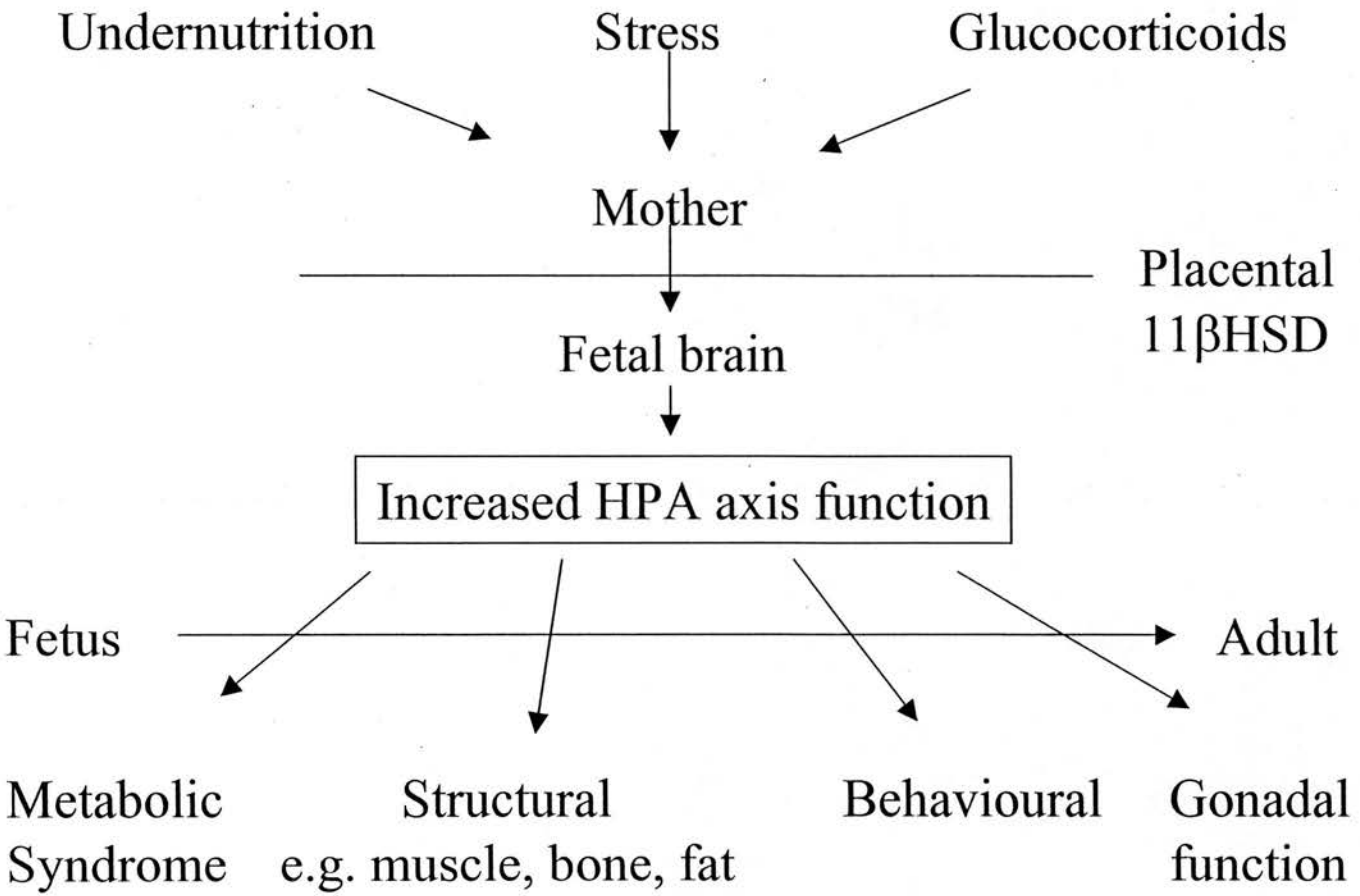


Figure 1.1 Programming of the HPA axis in animal models

responses, effects largely mediated via altered expression of glucocorticoid receptors in brain regions responsible for glucocorticoid feedback (Meaney *et al.* 1993).

1.3.3b Glucocorticoids and fetal growth

Glucocorticoids are essential for life, playing a key role in the regulation of fluid and electrolyte homeostasis, blood pressure, the immune system, metabolism and physiological responses to stress. During development, glucocorticoids are important in regulating fetal growth and organ maturation (e.g. stimulating surfactant production by the lungs), to prepare the fetus for extra-uterine life (Ballard 1979). However, excessive fetal exposure to glucocorticoids causes growth retardation (Reinisch *et al.* 1978) and human intrauterine growth retardation is associated with elevated cortisol levels (Goland *et al.* 1993). The fetus is normally protected from high maternal levels of glucocorticoids by placental 11 β-hydroxysteroid dehydrogenase type 2 (11 β-HSD2) (see 1.3.3c (iii)), which efficiently inactivates most maternal cortisol crossing the placenta (Benediktsson *et al.* 1993; Stewart *et al.* 1995). Activity of placental 11 β-HSD2 correlates with birthweight. The lowest activity, exposing the fetus to increased glucocorticoid levels from the maternal circulation, is seen in babies with the smallest birthweights (Stewart *et al.* 1995), thus suggesting fetal glucocorticoid load is a critical factor during development.

The mechanisms by which glucocorticoids retard fetal growth are poorly understood but are likely to involve other hormones and growth factors, particularly the IGFs. Glucocorticoids inhibit IGF production (Luo *et al.* 1989; Price *et al.* 1992; Li *et al.* 1993) and increase IGF binding protein (IGFBP) expression (Luo and Murphy 1990), thereby antagonising IGF action and retarding fetal growth. Interestingly, maternal high dose dexamethasone treatment induces fetal IGFBP-1 production (Price *et al.* 1992) and transgenic mice overexpressing IGFBP1 have low birthweight and develop hyperglycaemia in adulthood (Rajkuhmar *et al.* 1995). Thus the IGF system may be one pathway through which prenatal glucocorticoids could affect both birthweight and control of glucose metabolism. This also could be a common pathway for the programming effects of undernutrition as in sheep, maternal

undernutrition elevates cortisol (Fowden 1989) and suppresses fetal IGF-1 levels (Oliver *et al.* 1993)

Indeed, it is possible that the effects of maternal undernutrition are partly mediated by glucocorticoids. In the rat, maternal protein restriction attenuates placental 11β-HSD2 activity (Langley-Evans *et al.* 1996), weakening the placental barrier and significantly increasing fetal glucocorticoid load. Likewise, the hypertension induced in the offspring by a maternal low protein diet is prevented by pharmacological blockade of glucocorticoid biosynthesis and can be reintroduced by replacement of corticosterone (Langley-Evans 1997).

Together this evidence suggests glucocorticoids play a central role in mediating programming and that fetal glucocorticoid load is a critical factor in development. Before examining the consequences of fetal over-exposure to glucocorticoids (1.3.3f), the mechanisms of action of glucocorticoids will be described (1.3.3c). In addition, through describing how glucocorticoids themselves induce insulin resistance (1.3.3d) it will be seen that as well as mediating programming, variations in action of glucocorticoids may be an important factor underlying the pathogenesis of metabolic and cardiovascular disease (1.3.3e).

1.3.3c Mechanisms of glucocorticoid action

1.3.3c (i) The HPA axis (Figure 1.2)

Glucocorticoids (cortisol in humans and other mammals, corticosterone in rats and mice) are synthesized in the zona fasciculata/reticularis of the adrenal cortex under the regulation of the HPA axis. Adrenocorticotrophic hormone (ACTH) produced by corticotrophs in the anterior pituitary binds to adrenal cortex cell surface receptors coupled to adenyl cyclase. This rapidly activates the first, and rate-limiting step in the synthesis of steroid hormones i.e. removal of the cholesterol side-chain by side-chain cleavage enzyme to form pregnenolone (Figure 1.3).

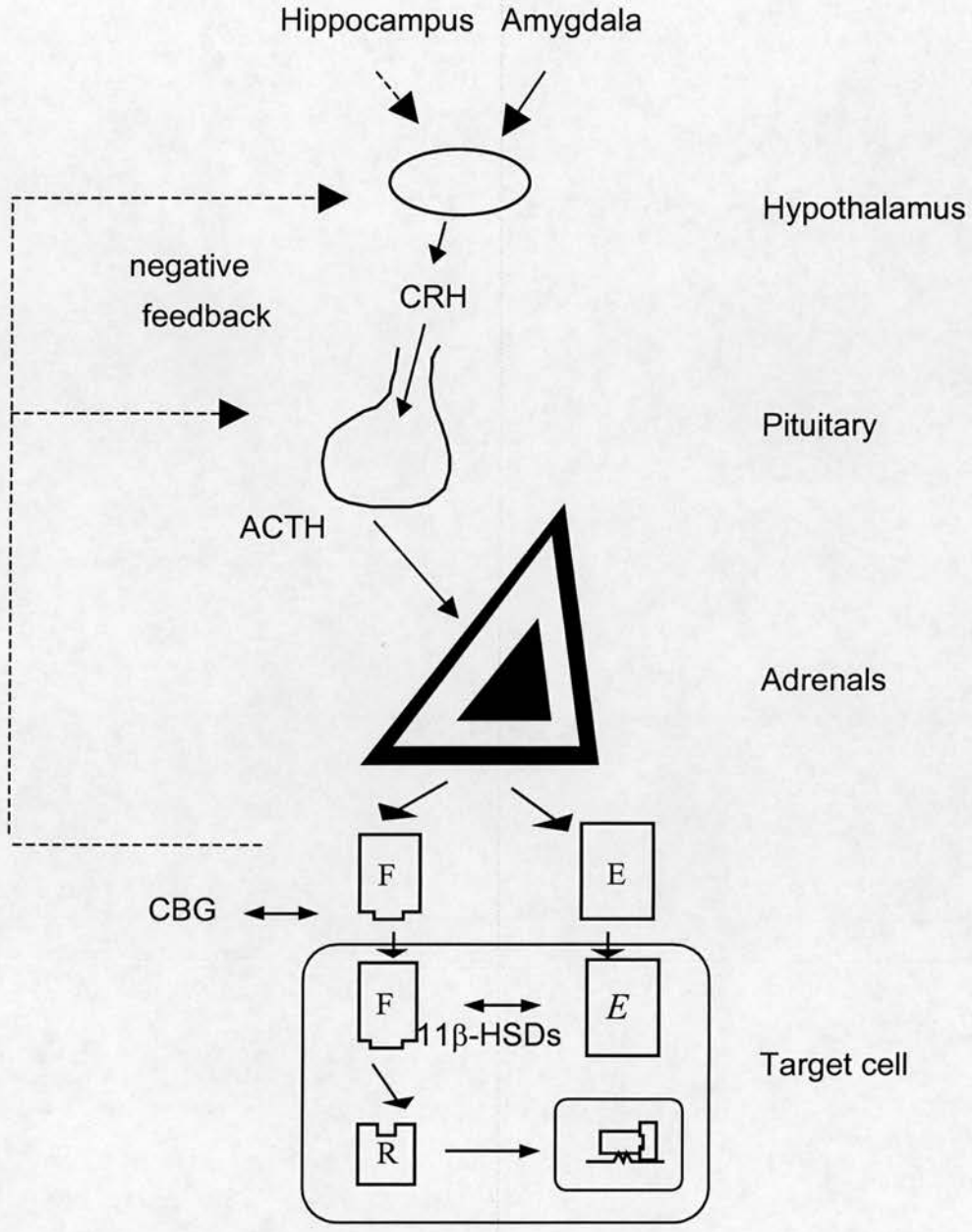


Figure 1.2 The hypothalamic-pituitary-adrenal (HPA) axis

Schematic representation of the HPA axis showing factors determining cortisol action. Cortisol (F), secreted by the adrenal gland under regulation of CRH (corticotrophin-releasing hormone) from the hypothalamus and ACTH (adrenocorticotrophic hormone) from the pituitary circulates bound to CBG (corticosteroid binding globulin). Free hormone diffuses into the target cell in which interconversion of cortisol and cortisone (E) by 11 β-HSDs regulates access of cortisol to receptors (R) and subsequent regulation of target genes, including those responsible for negative feedback.

- - - > inhibiting effect

⟶ stimulating effect

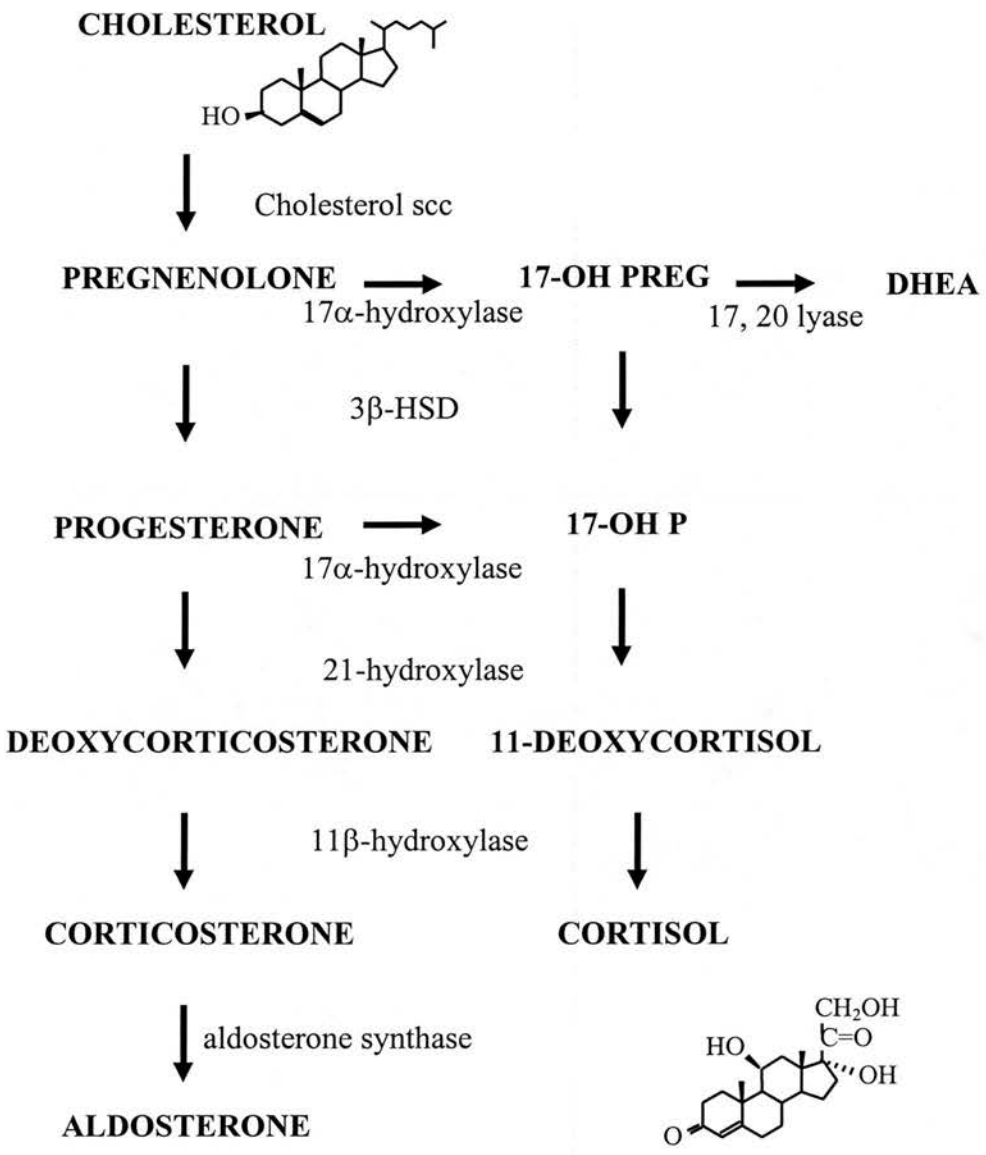


Figure 1.3 Steroid synthesis pathway

Cholesterol scc, cholesterol side-chain cleavage enzyme
17-OH PREG, 17-hydroxypregnenolone
17-OH P, 17-hydroxyprogesterone
DHEA, dehydroepiandrosterone

ACTH is regulated by hypothalamic corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP). In the resting state, basal levels of CRH, AVP, ACTH and cortisol are released in a pulsatile and circadian fashion, regulated by the suprachiasmatic nucleus of the hypothalamus, with plasma cortisol levels peaking prior to activity (i.e. in the morning in humans) and in response to 'stress'. Glucocorticoid secretion is auto-regulated by a negative feedback loop whereby glucocorticoids feedback to regulate secretion of CRH and ACTH at the level of the hypothalamus and pituitary respectively (Orth *et al.* 1992; Chrousos 1995). Higher centres, particularly the hippocampus, also play an important role in the negative feedback inhibition of glucocorticoid secretion (Feldman and Conforti 1980; Jacobson and Sapolsky 1991).

Circulating glucocorticoids are predominantly protein bound (Orth *et al.* 1992) to corticosteroid binding globulin (CBG) (70-75%) and albumin (15-20%), while 5-10% circulate as free steroid. Protein binding limits the concentration of free biologically active hormone and protects the hormone against degradation by P-450 enzymes in the liver.

1.3.3c (ii) Steroid receptors

As with other members of the steroid hormone family, glucocorticoids exert their actions by binding to intracellular receptors (Reviewed by Bamberger *et al.* 1996). Glucocorticoids bind with differing affinity to two types of receptor. The low affinity Type 2 or glucocorticoid receptor (GR) is widely distributed in brain and periphery, while the Type 1 or mineralocorticoid receptor (MR) has a high affinity for glucocorticoids but is more restricted in anatomical localization e.g. to distal nephron, colon and sweat glands.

GR and MR belong to the structurally-related nuclear receptor superfamily, which includes thyroid, retinoic and vitamin D receptors and a large group of orphan receptors for which no specific ligand has been identified. Nuclear receptors are highly specialised ligand-dependent transcription factors, which interact with specific

cis-acting elements to enhance or repress target gene expression. GR can also modulate gene expression by physically interacting with other transcription factors such as AP-1 and NF-κB.

1.3.3c (iii) 11 β-hydroxysteroid dehydrogenase enzymes (11 β-HSDs)

Access to steroid receptors is regulated by local activity of the 11 β-hydroxysteroid dehydrogenase enzymes (11 β-HSDs). These enzymes catalyse the interconversion of physiologically active glucocorticoids (cortisol in humans, corticosterone in rats) to inert 11-keto products (cortisone and 11-dehydrocorticosterone, respectively) (Murphy *et al.* 1974; Lopez-Bernal *et al.* 1980; Seckl 1993). 11 β-HSD2 in kidney inactivates cortisol, thus protecting MR from cortisol (Edwards *et al.* 1988; Funder *et al.* 1988; Brown *et al.* 1996). In contrast 11 β-HSD1 reactivates cortisol from inactive cortisone in many sites, ensuring adequate activation of GR (Jamieson *et al.* 1995).

1.3.3d Mechanisms of glucocorticoid induced insulin resistance

There are numerous potential sites of action for glucocorticoids to affect insulin sensitivity (Reviewed by Andrews and Walker 1999). The principal effects of glucocorticoids are to oppose the actions of insulin in the regulation of carbohydrate, lipid and protein metabolism by effects on the three main target tissues of liver, skeletal muscle and fat. Glucocorticoids increase blood glucose by mobilising substrates for hepatic gluconeogenesis. They stimulate release of amino acids from skeletal muscle, fatty acids and glycerol from adipose tissue, and increase expression of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEP-CK) (Hanson and Reshef 1997), so enhancing gluconeogenesis in the liver. Glucocorticoids also stimulate glycogen synthesis by activating glycogen synthase and inactivating the glycogen-mobilising enzyme glycogen phosphorylase. Peripheral glucose uptake and utilization is inhibited by glucocorticoids, partly due to decreased translocation of glucose transporters (GLUT 4) to the cell surface (Dimitriadis *et al.* 1997). The acute effects of glucocorticoids on fat metabolism are to stimulate lipolysis, providing gluconeogenic substrates (Boden 1997).

In addition to the metabolic effects of glucocorticoids on peripheral glucose uptake, glucocorticoids inhibit glucose-stimulated insulin secretion from pancreatic β-cells (Delauney *et al.* 1997). Non-metabolic effects of glucocorticoids include impairment of endothelium-dependent vasodilatation (Mangos *et al.* 2000), which is a potential mechanism to alter regional blood flow and thereby reduce glucose delivery to skeletal muscle.

The influence of glucocorticoids on insulin sensitivity is most apparent in clinical syndromes of cortisol excess or deficiency. In cortisol deficiency (hypopituitarism or hypoadrenalism (Addison's disease)), tissue sensitivity to insulin is increased. In contrast in cortisol excess (Cushing's syndrome), patients develop glucose intolerance, hypertension and central obesity, i.e. the cluster of risk factors for cardiovascular disease together described as the 'Metabolic Syndrome' (Reaven and Hoffman 1987). It is therefore an attractive idea that less profound disturbances of the HPA axis might underlie the Metabolic Syndrome and its link with obesity. Thus it has been proposed that excessive activity of glucocorticoids within the 'normal' physiological range, may contribute to the pathogenesis of insulin resistance and the development of cardiovascular risk factors (Björntorp *et al.* 1999).

1.3.3e Glucocorticoid activity in subjects with the Metabolic Syndrome

The hypothesis that cortisol contributes to the pathogenesis of essential hypertension and Type 2 diabetes mellitus was rejected by many on the basis of small case-control studies with very limited measurements of cortisol secretion (Huther and Sholz 1970; Vermeulen and Van der Straeten 1963). More recently, a series of studies have examined the relationship between aspects of cortisol secretion and tissue action and cardiovascular risk factors. In case-control and cross-sectional studies higher blood pressure, glucose intolerance, insulin resistance and hyperlipidaemia are associated with elevated cortisol concentrations in blood, saliva and urine (Watt *et al.* 1992; Filipovsky *et al.* 1996; Stolk *et al.* 1996; Phillips *et al.* 1998; Rosmond *et al.* 1998; Walker *et al.* 1998b; Fraser *et al.* 1999; Phillips *et al.* 2000) or impaired peripheral inactivation of cortisol (Walker *et al.* 1993; Soro *et al.* 1995; Walker *et al.* 1998b).

Men with cardiovascular risk factors also have altered peripheral sensitivity to glucocorticoids. Tissue sensitivity to cortisol is partly determined by the density of GR expression and can be assessed indirectly by measuring the intensity of dermal blanching after topical administration of synthetic glucocorticoids. The dermal vasoconstrictor response to glucocorticoids is increased in men with relative glucose intolerance and insulin resistance (Walker *et al.* 1998b), and also in men with essential hypertension (Walker *et al.* 1996), or with a familial predisposition to hypertension (Walker *et al.* 1998a). This response is also enhanced in healthy subjects who carry a polymorphism located with the restriction enzyme Bcl I within a non-coding part of the GR gene (Panarelli *et al.* 1998) which is associated with a familial predisposition to hypertension (Watt *et al.* 1992) and with greater hyperinsulinaemia (Weaver *et al.* 1992).

Together this evidence suggests that alterations in activity of the HPA axis whether by increased circulating levels of cortisol, increased GR sensitivity to cortisol or altered cortisol metabolism, may contribute to the pathogenesis of insulin resistance and the development of cardiovascular risk factors. Given the evidence from the epidemiological studies showing an association between low birthweight and the development of these cardiovascular risk factors, it has been proposed that glucocorticoids are a key link between low birthweight and the predisposition to diseases in adult life (Edwards *et al.* 1993; Seckl 1998). This hypothesis is supported by animal data and accumulating evidence from human studies.

1.3.3f Programming of the HPA axis

In rats, prenatal exposure to excess exogenous (dexamethasone) or endogenous (inhibition of 11-βHSD2 by carbenoxolone) glucocorticoids results in offspring of low birthweight and permanently elevated blood pressure and glucose and insulin levels in adulthood (Benediktsson *et al.* 1993; Lindsay *et al.* 1996). Such manipulations of the early environment 'programme' permanent changes in HPA axis regulation. These animals have impaired central negative feedback with decreased GR expression in the hippocampus, one of the most sensitive targets in rodents for glucocorticoid central negative feedback effects, resulting in increased

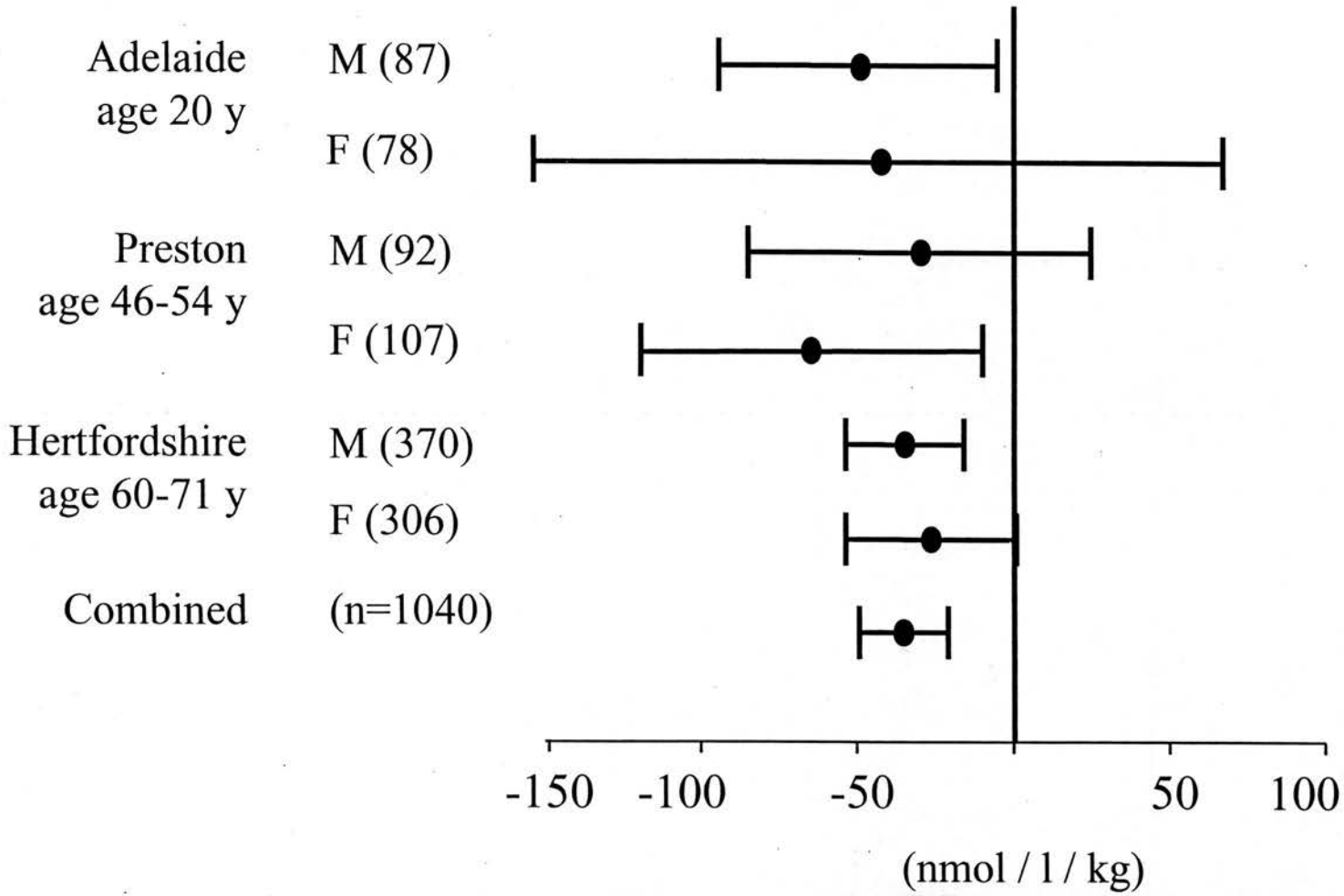
basal or stress-induced plasma glucocorticoid levels (Langley and Jackson 1994; Benediktsson *et al.* 1993; Levitt *et al.* 1996; Nyirenda *et al.* 1998). In contrast to the decreased central GR expression, peripheral (hepatic) GR expression is increased in association with up-regulation of the gluconeogenic enzyme PEP-CK (Nyirenda *et al.* 1998). One mechanism for tissue-specificity which allows differential GR regulation, is the use of multiple and tissue-specific promoters. In rat, the GR gene contains 8 coding exons (2-9) and at least 11 alternate untranslated exon 1 sequences (1_1-1_{11}), some of which are tissue-specific (McCormick *et al.* 2000). Importantly, tissue-specific first exon usage is altered by perinatal environmental manipulations (McCormick *et al.* 2000).

The evidence for programming of the HPA axis in humans is more limited. In a study of 64 year old men born in Hertfordshire, those who had lower birthweight had raised concentrations of fasting cortisol, a crude measure of cortisol secretion (Phillips *et al.* 1998). Mean fasting plasma cortisol concentrations fell progressively from 408 nmol/l among those whose birthweights were ≤ 5.5 lb (2.5 kg) to 309 nmol/l among those who weighed ≥ 9.5 lb (4.31 kg) at birth. Elevated plasma cortisol concentrations were also associated with higher blood pressure, plasma glucose and triglyceride concentrations (Phillips *et al.* 1998). These associations are also observed in men and women in early adult life and middle-age (Phillips *et al.* 2000) (Figure 1.4), and children and adolescents with lower birthweight have been reported to excrete more cortisol or its metabolites in urine (Clark *et al.* 1996; Harland *et al.* 1997).

These preliminary observations from human population studies, together with the data from animal studies, raised the possibility of an important link between birth size and adrenocortical activity in adult life. It has been proposed that events in early life permanently alter or 'programme' cortisol secretion and that this, together with increased obesity, leads to a high prevalence of the Metabolic Syndrome and cardiovascular disease in adult life. The aims of this thesis were to examine the aspects of the HPA axis and glucocorticoid action involved in programming and also the consequences of permanently altered glucocorticoid activity.

Figure 1.4 (from Phillips *et al* 2000) **0900 h plasma cortisol is elevated in men and women of low birthweight**

Change in fasting plasma cortisol concentration per kilogram increase in birthweight in populations of men and women 20 to 71 years of age in Adelaide, Preston and Hertfordshire. Regression coefficients and 95% confidence intervals derived from linear regression analysis of plasma cortisol on birthweight with adjustment for age and BMI.



1.4 Aims of thesis

A detailed study of the HPA axis was conducted in order to determine mechanisms underlying the elevated 0900 h plasma cortisol in men with low birthweight and/or the Metabolic Syndrome. Studies were designed to elucidate whether the elevated plasma cortisol was due to impaired central negative feedback, increased drive to cortisol secretion and / or delayed clearance of cortisol.

Feedback is traditionally tested using the dexamethasone suppression test, which assesses central negative feedback suppression of ACTH and cortisol secretion (Liddle 1960). Chapter 2 explores the use of this test with a very low dose of dexamethasone in order to reveal subtle variations in central feedback sensitivity, and investigates whether the test could be combined with measurement of salivary cortisol, which might be preferable as an epidemiological tool.

In Chapter 3 the responses of the HPA axis to dynamic testing, and whether the responses are linked with blood pressure or glucose intolerance are investigated in the Hertfordshire population in whom the original observations of an association between elevated 0900 h cortisol and low birthweight were made. Central negative feedback was tested using the very low dose dexamethasone suppression test described in Chapter 2 and responsiveness of the HPA axis to stimulation was tested using a very low dose short synacthen test. In addition, excretion of cortisol and its metabolites in urine was measured as an indication of clearance of cortisol.

Although the mechanisms underlying variations in plasma cortisol in subjects with the Metabolic Syndrome are unknown, it is known that plasma cortisol levels fall during an oral glucose tolerance test (OGTT) (Rodman and Bleicher 1973; Sober *et al.* 1977), and so this may be a useful tool for further determining acute differences in HPA axis activity in subjects with the Metabolic Syndrome. However, it is not known whether the fall in plasma cortisol reflects the circadian fall in circulating hormone or whether ingestion of glucose affects the response. In Chapter 4 this is determined in a placebo-controlled study of the effect of oral glucose on circulating plasma cortisol, in both glucose intolerant subjects and normal controls. The

findings of Chapter 4 enable interpretation of further measurements of cortisol during OGTTs in a larger cohort in Chapter 5. Here, whether the associations between cortisol and the Metabolic Syndrome are the same in women as in men are explored and the confounding effect of obesity is examined.

Little is known about peripheral sensitivity to glucocorticoids *in vivo* in man, partly due to limited accessibility of tissue for studying. Chapter 6 describes design of a novel competitive quantitative RT-PCR assay for measuring GR mRNA levels. This assay was used to quantitate GR expression in a rare collection of human skeletal muscle biopsies from the Uppsala Longitudinal Study of Adult Men (McKeigue *et al.* 1998), and to examine whether changes in GR expression in skeletal muscle were associated with features of the Metabolic Syndrome.

Finally, a potential mechanism for differential and tissue-specific regulation of GR expression (as has been observed in rats programmed by dexamethasone administration *in utero*) was examined in Chapter 7 by looking for alternate promoters of the human GR gene.

Chapter 2

Validation of the overnight very low dose dexamethasone suppression test

2.1 Introduction

Subtle increases in the circulating concentrations of glucocorticoids have been implicated in the aetiology of several common disorders including central obesity (Ljung *et al.* 1996), depression (Carroll *et al.* 1981), the Metabolic Syndrome (Björntorp 1993) and coronary artery disease (Brunner 1997). These changes in glucocorticoid exposure may be mediated by alterations in the sensitivity of the central feedback control of the HPA axis. These are demonstrable by administration of the synthetic glucocorticoid, dexamethasone at night, which results in the suppression of plasma cortisol concentrations the following morning.

The conventional protocol for the overnight dexamethasone suppression test (DST) (Liddle 1960) depends on the administration of between 1 and 2 mg dexamethasone and is designed to identify patients with Cushing's Syndrome. However, the use of a much lower dexamethasone dose, e.g. 0.25 mg (Best *et al.* 1997) may be more informative in distinguishing physiological variations in HPA axis activity. Recently it has been suggested that the very low dose test could be further refined by measuring the overnight suppression of salivary rather than plasma cortisol. Measurement of salivary cortisol levels offers several potential advantages enabling it to become a useful epidemiological tool for population studies of HPA axis function (Landon *et al.* 1982). These include simplicity of sample collection allowing the test to be performed at home, the avoidance of venepuncture which results in stress-induced hypercortisolaemia and that salivary measurements reflect free plasma cortisol levels which are less influenced by changes in CBG. Therefore, before conducting the field study of the HPA axis in Hertfordshire, the reproducibility of the very low dose DST was assessed by comparing cortisol suppression in saliva and plasma in 29 healthy subjects.

2.2 Methods

2.2a Clinical protocol

The study group consisted of 29 healthy subjects, 14 men and 15 women. None had a history of endocrine disease, or had received systemic or topical steroid treatment within the previous 6 months. The study was approved by the Southampton and South West Hampshire Health Authority and University of Southampton Joint Ethical Committee.

In each subject two very low dose DSTs were performed at least one week but no more than ten weeks apart. On the first day the subjects attended after fasting for 12 h and saliva and blood samples were collected at 0830 h. Between two and four ml of saliva was collected into a salivette tube (Sarstedt Ltd., Leicester, U.K.). The subjects took 0.25 mg dexamethasone at 2200 h the same night and attended the following morning after a further 12 h overnight fast for collection of saliva and blood samples at 0830 h. Plasma and saliva samples were separated by centrifugation and stored at -80° C until analysis. The subjects also completed the 10 item General Health Questionnaire (cut off score for depression = score > 5) a previously validated questionnaire for detecting depression (Yesavage *et al.* 1983 and see Appendix A1 question 9).

2.2b Anthropometry

The subjects' height was measured with a portable stadiometer (CMS weighing equipment, Camden, London, U.K.) to the nearest 0.1 cm, avoiding digit-preference. Subjects were measured standing upright and without shoes, with head placed in the Frankfurt Plane, such that an imaginary line joining the upper margin of the external auditory meatus and the lower border of the orbit of the eye was horizontal. Weight was measured with a portable SECA scale (SECA Ltd., Birmingham, U.K.), without shoes and in lightweight clothing, to the nearest 0.1 kg. Body mass index (BMI) was calculated as the weight (kg) divided by the height (m) squared.

Waist and hip circumferences were measured with a steel tape measure. Waist was measured with the individual standing in the upright position, in a horizontal plane midway between the superior iliac crest and the ribcage in the mid-axillary line, at the end of normal expiration, to the nearest 0.1 cm. Hip circumference was measured as the largest horizontal circumference at the maximum protrusion of the buttocks with the feet together. The waist to hip ratio (WHR) was calculated as a measure of central obesity.

2.2c Laboratory measurements

Cortisol was measured in plasma by radioimmunoassay (Guildhay antisera, Moore *et al.* 1985). Cortisol and cortisone were measured in saliva by time-resolved immunofluorescent assay ('DELFIA', Wood *et al.* 1997). The coefficients of variation (CV) were 6.3% at 300 nmol/l for plasma and 8.5% at 12 nmol/l for saliva assays.

2.2d Statistical analysis

As the distributions of cortisol measurements were highly skewed, $\log_e$-transformed values were used in all analyses. Bland-Altman plots (the differences between two repeated measurements for each subject against the mean of those measurements) were produced for post dexamethasone plasma and salivary cortisol measures (Bland and Altman 1986). From these the 95% range for the differences about their mean was calculated and this used as an indication of repeatability. Fractional suppression of cortisol in plasma and saliva was calculated as (day 1 sample – day 2 sample) / day 1 sample (%). Analysis advised by statistician HE Syddall, Southampton.

2.3 Results

The subjects were aged 24 - 54 (mean 35.1) years with BMI ranging from 17.9 to 31.3 (means 27.1 in men vs. 23.5 in women, p=0.001) kg/m^2 and WHR ranging from

0.82 to 1.20 (mean 0.94) in men and 0.67 to 0.81 (mean 0.74) in women. None of the subjects was depressed as indicated by the General Health Questionnaire. Table 2.1 shows the baseline and post dexamethasone suppression concentrations of cortisol measured in plasma and saliva.

Table 2.1. Mean (95% confidence interval) fasting plasma cortisol and salivary cortisol concentrations before and after suppression with 0.25 mg dexamethasone in duplicate tests carried out in 29 subjects at least one week apart

	Baseline		First phase		Second phase	
	Mean (95% CI)	Range	Mean (95% CI)	Range	Mean (95% CI)	Range
Plasma cortisol (nmol/l)	407.5 (362.3, 458.3)	232-958	270.4 (231.2, 316.3)	116-802	265.1 (226.6, 310.1)	105-853
Salivary cortisol (nmol/l)	15.3 (13.11,17.94)	4.4-34.4	8.6 (6.65,11.08)	1.6-29.2	7.3 (5.45,9.82)	1.6-23.8

The basal plasma cortisol concentrations ranged from 232 to 958 nmol/l (mean 270.4 nmol/l) and tended to be lower in the men than the women (371.4 vs 485.6 nmol/l, p=0.06). The mean concentration of cortisol measured after dexamethasone suppression was similar for both tests as assessed by plasma (270.4 vs 265.1 nmol/l) or saliva measurements (cortisol 8.6 vs. 7.3 nmol/l, total cortisol + cortisone, 37.6 vs. 29.4 nmol/l). Also the fractional suppression of plasma cortisol, which was similar on the two study phases (30.6% and 30.4%), was somewhat but not significantly greater than the fractional cortisol suppression measured in saliva (27.3% and 28.8%). Neither the fractional suppression nor the post dexamethasone cortisol measurements differed according to sex, age, BMI or WHR.

The reproducibility of the post dexamethasone cortisol concentrations obtained from the two tests is illustrated by the Bland-Altman plots in Figures 2.1 and 2.2 (showing the differences in cortisol concentration at the two study phases plotted against the mean). For plasma (Figure 2.1), the mean difference did not differ significantly from zero (mean 0.02, $p = 0.80$). 95% of differences were within 0.78 $\log_e$ units from the mean (-0.76 to +0.80) indicating that a repeated plasma measurement could be approximately half as small or twice as large as the first. For saliva (Figure 2.2), the mean also did not differ significantly from zero (mean 0.16, $p = 0.29$), but 95% of differences were within 1.64 $\log_e$ units from the mean (-1.48 to +1.80), thus indicating that a repeated saliva measurement could be approximately five times as small or five times as large as the first. Saliva repeatability was no better when cortisone was considered, either alone, when as a total of salivary cortisol and cortisone, or as a ratio of measurements.

Figure 2.1 Differences in $\log_e$ (post-dexamethasone plasma cortisol) concentrations plotted against mean $\log_e$ concentration, n=29

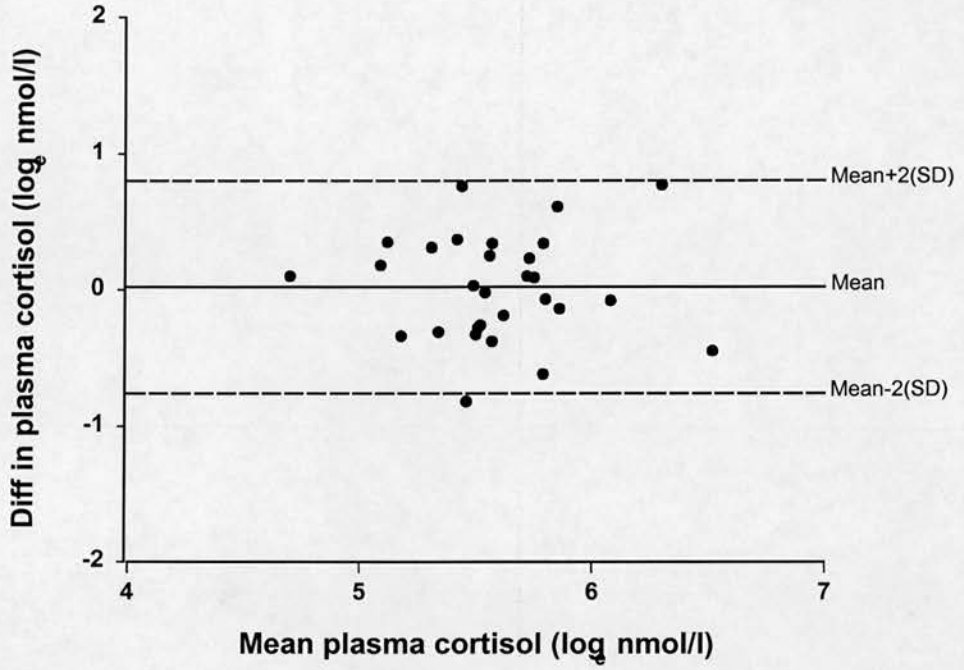
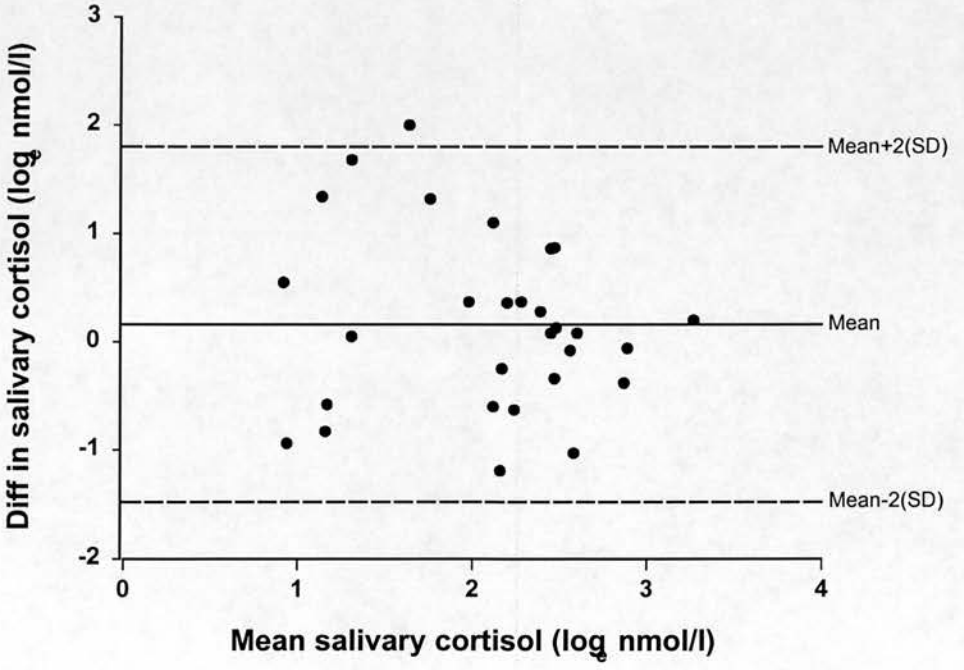


Figure 2.2 Differences in $\log_e$ (post dexamethasone salivary cortisol) concentrations plotted against mean $\log_e$ concentration, n=29



2.4 Discussion

The results suggest that the use of salivary cortisol measurement in the analysis of a very low dose overnight DST is less reproducible than plasma cortisol measurement. For saliva the repeated measurement was as much as five times as small or five times as large as the first for 95% of the time, while for plasma a repeated measurement was only half as small or twice as large as the first.

Although the DST has gained increasing importance in studies examining the role of abnormalities of the HPA axis in pathogenesis of several common diseases, there is surprisingly little information on the reproducibility of the test. Charles *et al.* (1982) showed similar levels of HPA axis suppression in 17 of 19 depressed patients studied on two occasions after the administration of 1 mg dexamethasone. A similar conclusion was reached by Greden *et al.* (1983), who showed that 9 of 13 subjects studied on two occasions had the same abnormal response to 1 mg dexamethasone, defined by a threshold level of plasma cortisol suppression. However, in these studies of subjects with clear evidence of disease, no data was given on the reproducibility of the test within individuals. Importantly the use of such a high dose of dexamethasone would be expected to completely suppress the axis and hence be more likely to result in reproducibly undetectable cortisol levels. This contrasts with this study where post dexamethasone cortisol levels were well within the detection limits of the assay showing an approximately 30% suppression and hence a greater within-subject variability.

The poorer performance of the 0.25 mg DST based on salivary measurements is difficult to explain. It was not due to assay imprecision as the 'DELFIA' assay used has been shown to be reproducible over a wide range of cortisol concentrations and the assays for saliva and plasma had similar CVs. In addition, previous studies have shown that saliva quantitatively reflects free plasma cortisol, and therefore might be expected to be a better index of dexamethasone suppression. Also, although salivary cortisol is converted to cortisone by 11β-HSD2 (Walker and Best 1995) the salivary results were not significantly improved when the total of cortisol and cortisone

concentrations was considered. Thus the reason for the poor reproducibility of the saliva measurement is unclear but may include sampling problems (e.g. variable salivary flow rates) or a poor correlation between salivary and plasma cortisol concentrations at low (post dexamethasone) concentrations.

These data imply that the intrinsic variation in salivary cortisol levels has been underestimated. Because of the poorer repeatability of salivary measurements as compared to plasma it was elected to measure cortisol concentrations in plasma following dexamethasone suppression in the field study.

Chapter 3

Dynamic testing of the HPA axis in men with low birthweight and cardiovascular risk factors

3.1 Introduction

The mechanisms underlying the association of Type 2 diabetes mellitus, raised blood pressure and dyslipidaemia (i.e. the Metabolic Syndrome), and their exacerbation by obesity, are not known, but are important in our understanding of the pathophysiology of atheromatous disease. Since patients with Cushing's syndrome develop these clinical features, it is an attractive idea that less profound disturbances of the HPA axis might underlie the Metabolic Syndrome and its link with obesity.

In animal models, manipulation of the early environment 'programmes' permanent changes in HPA axis regulation: rats exposed to glucocorticoids *in utero* have impaired central negative feedback with decreased hippocampal GR expression and increased basal or stress-induced plasma glucocorticoid levels (Levitt *et al.* 1996). In studies of adult men higher fasting morning plasma cortisol concentrations, a crude measure of cortisol secretion, are associated with higher blood pressure, plasma glucose and triglyceride concentrations, and lower birthweight (Phillips *et al.* 1998; Phillips *et al.* 2000). These studies have led to the hypothesis that events in early life permanently alter or 'programme' cortisol secretion and that this, together with increased obesity, leads to a high prevalence of the Metabolic Syndrome and cardiovascular disease in adult life.

The aim was to test this hypothesis in the Hertfordshire population in whom the observations of an association between elevated 0900h plasma cortisol and low birthweight and / or the Metabolic Syndrome were made. The study was designed to characterise abnormalities of cortisol secretion in relation to features of the Metabolic Syndrome, obesity, and birthweight.

3.2 Methods

3.2a Subjects

In 1991, 370 men born between 1920 and 1930 in East Hertfordshire, and still resident there, underwent 75-g oral glucose tolerance tests (OGTTs) and blood pressure measurements as part of a study examining the relationships between early life events and subsequent glucose tolerance (Hales *et al.* 1991). Plasma cortisol was analysed in the fasting samples from this study in 1997 (Phillips *et al.* 1998). In 1997, the General Practitioners of the surviving 245 men were approached and asked if they would be willing for their patients to be contacted again. Subjects were contacted by letter and invited to take part in a cross-sectional study examining the relationship between the early environment and the HPA axis. Subjects with clinical evidence of pituitary or adrenal disease and those who had received oral glucocorticoids in the previous 3 months were excluded. 205 men were suitable and agreed to take part. Ethical approval was obtained from the East and North Hertfordshire Health Authority Local Research Ethics Committee and written informed consent was obtained from each subject.

3.2b Interview

The subjects were visited at home by one of four trained MRC nurses who administered a questionnaire (see Appendix A 1). Information about medical and social history, family history of diabetes and hypertension, smoking habits, alcohol consumption and current medication was recorded. Mood was assessed by the 10 item General Health Questionnaire (Yesavage *et al.* 1983). The subjects were then invited to attend a morning clinic at Hertford County Hospital for dynamic suppression and stimulation tests of the HPA axis. They were given a tablet of 0.25 mg dexamethasone with instructions to take this at 2200 h on the night before the clinic and to fast overnight.

3.2c Clinical protocol

(i) Dexamethasone/ACTH$_{1-24}$ test

Having taken 0.25 mg dexamethasone at 2200 h and fasted overnight, subjects attended clinic the following morning. At 0830 h, a 21-gauge butterfly cannula was inserted in an antecubital fossa vein. After 30 min rest, a baseline 10 ml venous blood sample was collected into a lithium heparin vacutainer before 1 μg of freshly diluted ACTH$_{1-24}$ (tetracosactrin, Synacthen, Alliance, Chippenham, U.K.) was injected as a bolus with a 10 ml 0.9% NaCl flush. The dilute solution of synacthen was prepared by adding 250 μg to 50 ml of 0.9% NaCl in a 50 ml syringe. The resulting solution (5 μg/ml) was mixed and used immediately after preparation. 10 ml venous blood samples were collected through the cannula at 20, 30, 40 and 60 min after ACTH$_{1-24}$ administration. Samples were centrifuged immediately at 3,000 rpm for 20 min and plasma stored at –80°C for subsequent hormone analysis.

Dexamethasone (0.25 mg) and ACTH$_{1-24}$ (1 μg) doses were selected to provide an average 50-75% of maximal suppression or stimulation, respectively, with a wide range (Best *et al.* 1997; Daidoh *et al.* 1995). More conventional doses (e.g. 1 mg dexamethasone or 250 μg ACTH$_{1-24}$) would be expected to produce maximal effects in all of these otherwise healthy participants and would not detect subtle alterations in the control of cortisol secretion.

(ii) 24 h urine collection

At the preliminary interview, subjects were given written and oral instructions for a 24 h urine collection to be collected a week before or after the dexamethasone/ACTH$_{1-24}$ test. Standard bottles (of known weight) were issued and collected by the nurses at a pre-arranged date. The urine collection was weighed, the weight of the bottle subtracted and the total volume recorded in ml. Two 20 ml aliquots were labelled and stored at -20°C for analysis of cortisol and its metabolites.

3.2d Anthropometry

Anthropometric measurements were recorded at the end of the clinic. As previously described (Chapter 2.2b), height was measured using a portable stadiometer and weight was measured with a portable SECA scale. BMI was calculated as the weight (kg) divided by the height (m) squared. Waist and hip circumferences were measured with a steel tape measure and WHR calculated as a measure of central obesity.

Two inter/intra-observer studies for recording height, weight, waist and hip measurements were conducted before and between clinics to ensure repeatability of these measurements, both between and within individual staff. 12 subjects were measured twice by each of the 4 nurses, who were blinded to their own and others' measurements of each subject. There were no significant differences in the height, weight, waist or hip measurements made between nurses.

3.2e Laboratory meaurements

(i) Plasma analysis

Plasma cortisol was measured by RIA as before (see 2.2c) in samples at all time points (T = 0, 20, 30, 40 and 60 min). RIAs were used to measure corticosteroid-binding globulin (CBG) (Medgenics Diagnostics, Fleurus, Belgium) and dexamethasone levels (enzyme-linked immunosorbent assay adapted from Cozart Biosciences Ltd., Abingdon, Oxford) in the first sample (T = 0). In a subgroup of men with contrasting birthweight (>9.5 lb (4.31 kg) or ≤6.5 lb (2.92 kg), n=12 in each group), plasma dehydroepiandrosterone (Diagnostic Systems Laboratories, Inc., Webster, Texas, USA), 17-α-hydroxyprogesterone (in-house RIA) and progesterone ("Immulite" analyser, Diagnostic Products Ltd, Gwynedd, Wales) were measured. Measurements of glucose, triglyceride and insulin concentrations which have been reported previously (Hales *et al.* 1991) were used in the analysis.

(ii) Urine analysis

Cortisol, cortisone and their metabolites (see Figure 3.1) were measured in urine by gas chromatography/electron impact mass spectrometry following Sep-pak C18 extraction, hydrolysis with β-glucuronidase, and formation of the methoxime-trimethylsilyl derivatives. Epi-cortisol and epi-tetrahydrocortisol were used as internal standards (Best *et al.* 1997; Best and Walker 1997). Total cortisol and metabolite excretion was calculated as tetrahydrocortisols (THFs) + tetrahydrocortisone (THE) + cortols + cortolones. Ratios of urinary metabolites were used to infer relative activation of the principle enzymes metabolising cortisol. Relative reduction by 5α- and 5β-reductases was inferred from the 5β-THF/5α-THF ratio. Total A-ring reduction of cortisol was inferred from the ratio of cortisol/THFs and 5β-reductase activity from the ratio of cortisone/THE. Whole-body equilibrium between cortisol and cortisone, determined by the balance of tissue-specific activities of 11β-reductase and 11β-dehydrogenase activities, was inferred from the ratio of THFs/THE. Renal 11β-dehydrogenase activity was inferred from the urinary free cortisol/cortisone ratio.

Urine creatinine was measured using the Jaffé reaction on the Bayer PLC (Newbury, UK) "Advia" analyser.

3.2f Statistical analysis

To obtain normally distributed variables, measurements of glucose, triglycerides, urinary cortisol metabolites, peak cortisol following $ACTH_{1\text{-}24}$, and area under the curve after $ACTH_{1\text{-}24}$ for dehydroepiandrosterone and 17-α-hydroxyprogesterone, were $\log_e$-transformed. Geometric means and sds are therefore presented for these variables. Associations between continuously distributed variables were assessed by the Pearson correlation coefficient, and associations between continuous and categorical variables by the Mann-Whitney U-test or the two-sample t-test as appropriate. Multiple linear regression was then used to explore the relationship between continuously distributed response variables and possible explanatory variables, with adjustment for confounding factors. Multiple logistic regression was

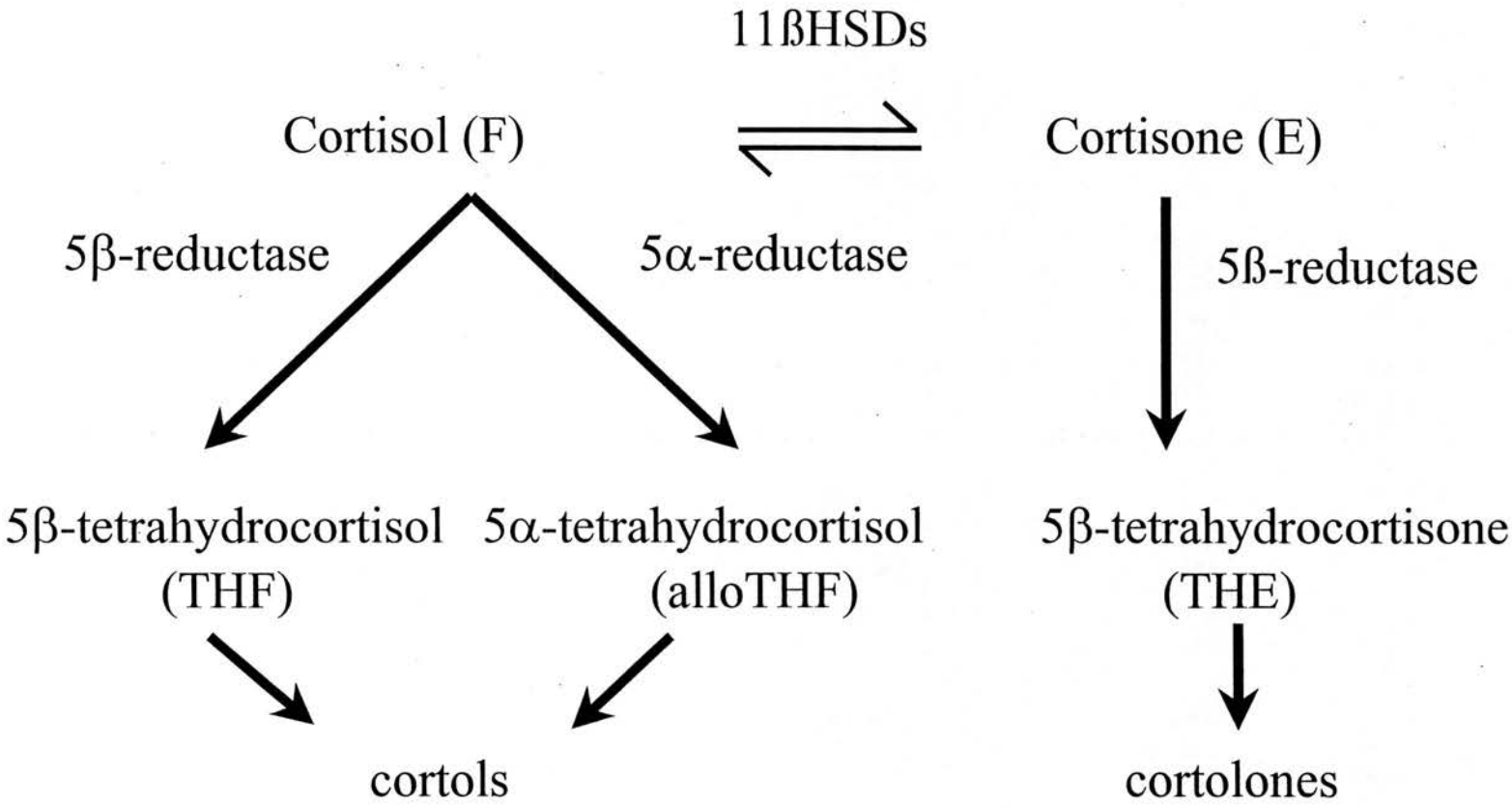


Figure 3.1 Urinary cortisol and cortisone metabolites

used to analyse binary response variables. In addition to analysing the peak cortisol response to $ACTH_{1-24}$ using the methods described above, a longitudinal analysis of the cortisol response to $ACTH_{1-24}$ was also conducted. The longitudinal approach considers the full series of cortisol data for each subject and models the average response during the test in relation to the factors of interest, taking into account the effects of time, and the autocorrelation of cortisol measurements within each subject (Zeger and Liang 1996). All statistical analysis was carried out by statistician HE Syddall, Southampton, using STATA, release 5; the xtgee feature was used to implement the longitudinal analysis (Statacorp 1997, Stata Statistical Software Release 5, College Station, TX: Stata Corporation).

3.3 Results

3.3.1 Subject characteristics and potential confounders

The men were aged between 66 and 77 (mean 70.9, sd 3.1) years with a mean BMI of 26.9 (sd 3.7) kg/m^2. Fifteen men had Type 2 diabetes mellitus (2 h glucose $\geq$ 11.1 mmol/l) and 33 had impaired glucose tolerance (IGT) (2 h glucose 7.8 to 11.0 mmol/l). Mean systolic blood pressure was 161.5 mmHg (sd 22.1 mmHg) and 73 men were receiving treatment with antihypertensive drugs. Two subjects were excluded from analysis of plasma cortisol concentrations because of extreme values: one had a vaso-vagal event after intravenous cannulation and the other was receiving ethinyloestradiol treatment. Six men had missing values for glucose measurements. None of the measurements of cortisol in plasma or urine correlated with age or differed in subjects receiving topical or inhaled corticosteroid therapy (n=16). None of the differences in plasma cortisol described below were accounted for by variation in plasma CBG or dexamethasone concentrations.

3.3.1a Obesity

Obesity, reflected in increased BMI, was associated with higher blood pressure (r=0.18, p=0.04), hypertriglyceridaemia (r=0.28, p=0.0001) and glucose intolerance

(r=0.16, p=0.02). Obesity was also associated with a linear increase in total urinary cortisol metabolite excretion (r=0.19, p=0.006) but did not predict plasma cortisol after dexamethasone or $ACTH_{1-24}$. Central obesity, reflected in increased WHR, was associated with similar trends. In addition, increased WHR predicted marginally lower plasma cortisol following 0.25 mg dexamethasone (r=-0.13, p=0.06) and disproportionately higher excretion of 5α- rather than 5β-reduced metabolites of cortisol (r=-0.14, p=0.05). Obesity was not associated with altered ratios of cortisol/cortisone metabolites. Increased lean body mass, as judged by urinary creatinine excretion (Malina 1986) was also associated with higher total urinary cortisol metabolites (r=0.23, p=0.001). Neither the effects of obesity nor urinary creatinine were independent predictors of total urinary metabolite excretion in multiple regression analysis. Urinary creatinine was not associated with plasma cortisol concentrations.

3.3.1b Depression and social class

Men with a current or previous history of depression (n=12) had greater peak plasma cortisol concentrations after $ACTH_{1-24}$ (474.0 (sd 1.1) versus 428.7 (sd 1.2) nmol/l, p=0.03) and higher total urinary cortisol metabolites (median (inter-quartile range) 22.4 (16.3, 48.2) versus 17.8 (11.3, 24.8) mg/24h, p=0.04). Men with manual occupations (class IIIM-V, n=134) had no difference in plasma cortisol concentrations, but excreted less total cortisol metabolites than men with non-manual occupations (class I-IIIN, n=69) (15.9 (sd 2.1) versus 20.8 (1.8) mg/24h, p=0.008).

3.3.2 Associations with birthweight

Table 3.1 shows relationships between birthweight and cortisol and its metabolites. Lower birthweight was associated with a greater rise in plasma cortisol concentrations after $ACTH_{1-24}$ and a later peak time (Figure 3.2), but no difference in plasma cortisol after dexamethasone. The inverse relationship between birthweight and adrenal $ACTH_{1-24}$ responsiveness remained after exclusion of men with IGT and Type 2 diabetes mellitus and/or treated hypertension, and was not confounded by obesity.

Table 3.1

Relationships between birthweight and cortisol and its metabolites

Birthweight in pounds (kg)	*No. of men*	*Post-dexamethasone plasma cortisol (nmol/l)* [a]	*Peak plasma cortisol (nmol/l)* [b,c]	*Total urinary cortisol metabolites (mg/24h)* [b]	*Total urinary cortisol metabolites per creatinine excretion (mg/mmol)* [b]
≤6.5 lb (2.92)	26	185.3 (88.7)	458.2 (1.2)	22.8 (1.7)	7.24 (1.7)
-7.5 lb (3.41)	59	208.2 (92.9)	436.1 (1.2)	15.4 (2.2)	3.74 (2.2)
-8.5 lb (3.86)	73	202.0 (94.9)	417.0 (1.2)	17.3 (2.2)	4.10 (2.1)
-9.5 lb (4.31)	33	210.2 (98.2)	444.5 (1.2)	14.1 (1.9)	3.35 (2.0)
>9.5 lb (4.31)	12	189.3 (80.2)	405.3 (1.2)	23.6 (1.7)	5.26 (1.7)
All	203	202.2 (92.8)	431.3 (1.2)	17.1 (2.1)	4.22 (7.8)
		ns	*p = 0.03*[d]		*p = 0.10*[d]
			p = 0.03[e]		*p = 0.04*[e]
				p = 0.03[f]	*p = 0.07*[f]

[a] arithmetic mean (sd) for 0900 h plasma cortisol after 0.25 mg dexamethasone
[b] geometric mean (sd)
[c] peak plasma cortisol post 1 μg $ACTH_{1-24}$ calculated from individual maximums rather than mean data in Fig. 3.1
[d] *p* = unadjusted *p*-value
[e] *p* = *p*-value adjusted for BMI, depression and social class
[f] *p* = unadjusted *p*-value for quadratic trend

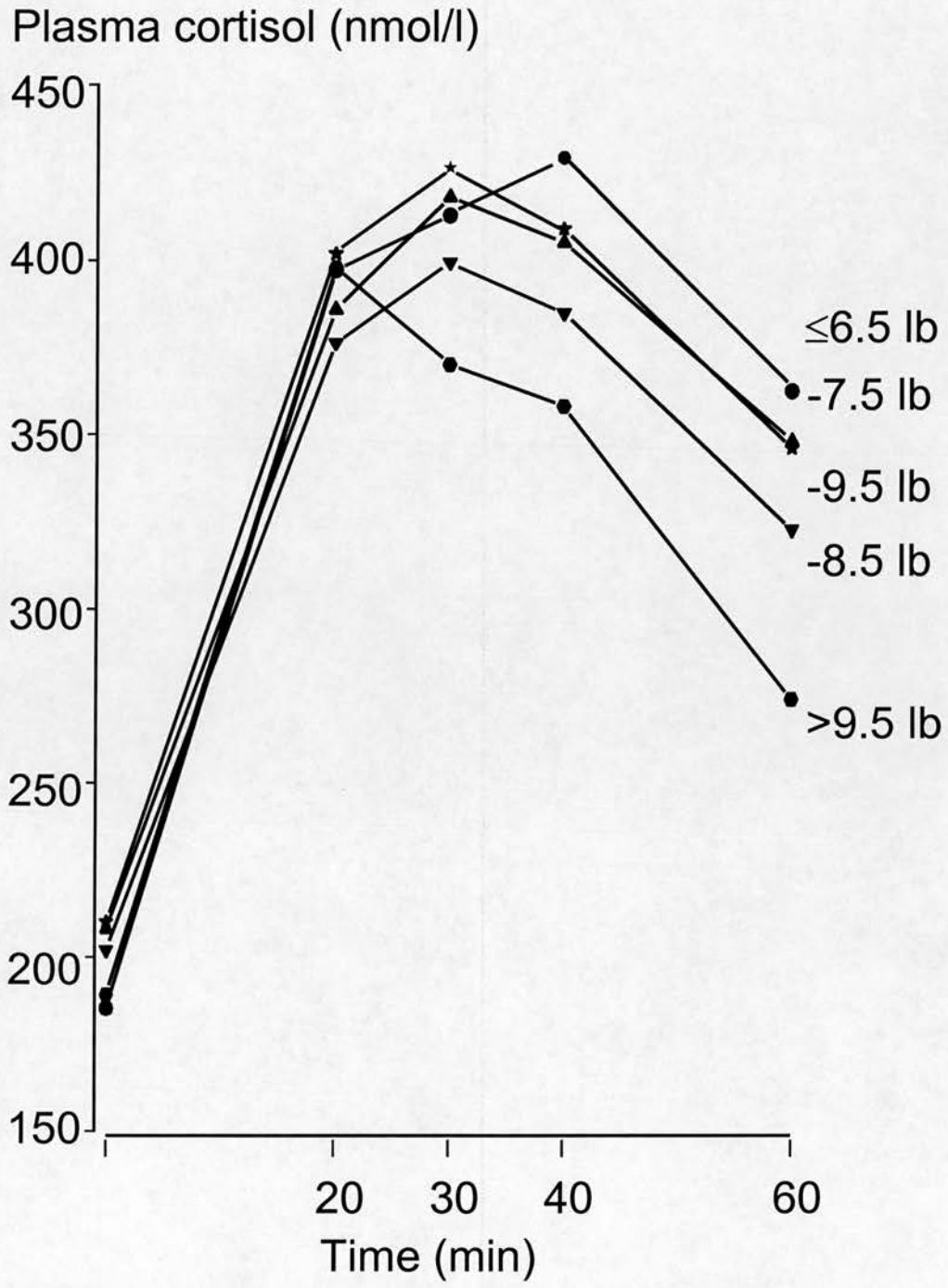


Figure 3.2
Plasma cortisol profiles during dexamethasone/ACTH$_{1-24}$ test according to birthweight

p=0.03 from longitudinal analysis, interaction of cortisol response by birthweight with time

● birthweight ≤ 6.5lb (2.92kg); ▲ -7.5lb (3.41kg); ▼ - 8.5lb (3.86kg); ★ - 9.5lb (4.31kg);
⬢ >9.5lb (4.31kg)

Differences in adrenocortical responses to $ACTH_{1-24}$ in a subgroup of men with contrasting birthweight (>9.5 lb (4.31 kg) or ≤6.5 lb (2.92 kg), n=12 in each group) were further explored by measurement of other ACTH-dependent adrenal steroids in plasma. Men with lower birthweight also had higher levels of dehydroepiandrosterone (mean area under curve: birthweight ≤6.5 lb 6.5 (sd 2.2) nmol/l.hrs; >9.5 lb 4.9 (sd 1.5) nmol/l.hrs), 17-α-hydroxyprogesterone (birthweight ≤6.5 lb 9.7 (sd 1.3) nmol/l.hrs; >9.5 lb 8.1 (sd 1.3) nmol/l.hrs) and progesterone (birthweight ≤6.5 lb 3.6 (sd 0.5) nmol/l.hrs; >9.5 lb 3.1 (sd 0.7) nmol/l.hrs) indicating that no common biosynthetic defect, such as 21-hydroxylase deficiency, accounts for the difference in cortisol response.

Total urinary cortisol metabolite excretion was higher in men with the lowest and highest birthweights (p=0.03 for quadratic trend). Although higher birthweight men were not more obese, they were taller and heavier as adults (r=0.23, p=0.001) and excreted more creatinine in urine (r=0.20, p=0.006), which could confound the relationship with urinary glucocorticoid excretion. After adjustment for urinary creatinine and obesity (either BMI or WHR) an inverse linear relationship, rather than a quadratic trend, was evident such that low birthweight men excreted more urinary cortisol metabolites (r=-0.29, p=0.04). Birthweight was not associated with cortisol metabolite ratios.

3.3.3 Associations with features of the Metabolic Syndrome

Table 3.2 shows relationships between plasma cortisol and urinary cortisol metabolites with blood pressure, glucose tolerance and fasting plasma triglyceride concentrations, after correction for the potential confounding effects of obesity, depression, social class, and urinary creatinine where appropriate. Plasma cortisol concentration at 0900 h following dexamethasone was not associated with cardiovascular risk factors. However, peak plasma cortisol concentration following $ACTH_{1-24}$ was higher in men with higher blood pressure and higher fasting plasma triglyceride concentrations and tended to be higher in men with higher post-glucose plasma glucose concentrations. Likewise, in a longitudinal analysis of the cortisol

profiles there were similar positive associations between mean plasma cortisol concentration and these features, with or without adjustment for potential confounding factors. In men with all three features of the Metabolic Syndrome (previously defined (Barker *et al.* 1993b) as systolic blood pressure >160 mmHg or subject receiving anti-hypertensive therapy (n=120); the presence of IGT or Type 2 diabetes mellitus (n=48); fasting plasma triglyceride >1.4 mmol/l (n=101)), peak and mean plasma cortisol concentration over time were significantly elevated. Total urinary cortisol metabolite excretion also tended to be greater in men with these cardiovascular risk factors.

3.3.4 Predictors of the Metabolic Syndrome

Having examined individual features of the Metabolic Syndrome, logistic regression modelling was then performed to identify predictors of combined features of the Metabolic Syndrome (as defined above). Potential variables included age, social class, birthweight, WHR, BMI, plasma cortisol after dexamethasone, peak plasma cortisol after $ACTH_{1-24}$, total urinary cortisol metabolite excretion, and ratio of 5β-/5α-reduced metabolites of cortisol. The best fitting model identified effects of BMI (p=0.003), peak plasma cortisol after $ACTH_{1-24}$ (p=0.03) and birthweight (p=0.02). The effect of birthweight was more significant when peak plasma cortisol was excluded (p=0.008), and the effect of peak plasma cortisol was more significant when birthweight was excluded (p=0.02) (see Figure 3.3). The estimated odds ratios (95% CI) for the 'Metabolic Syndrome' are 1.18 (1.06-1.32) for a unit increase in BMI, 1.55 (1.09-2.21) for a one pound decrease in birthweight, and 1.28 (1.02-1.61) for a 50 nmol/l increase in peak cortisol concentration. Effects of WHR were similar to BMI.

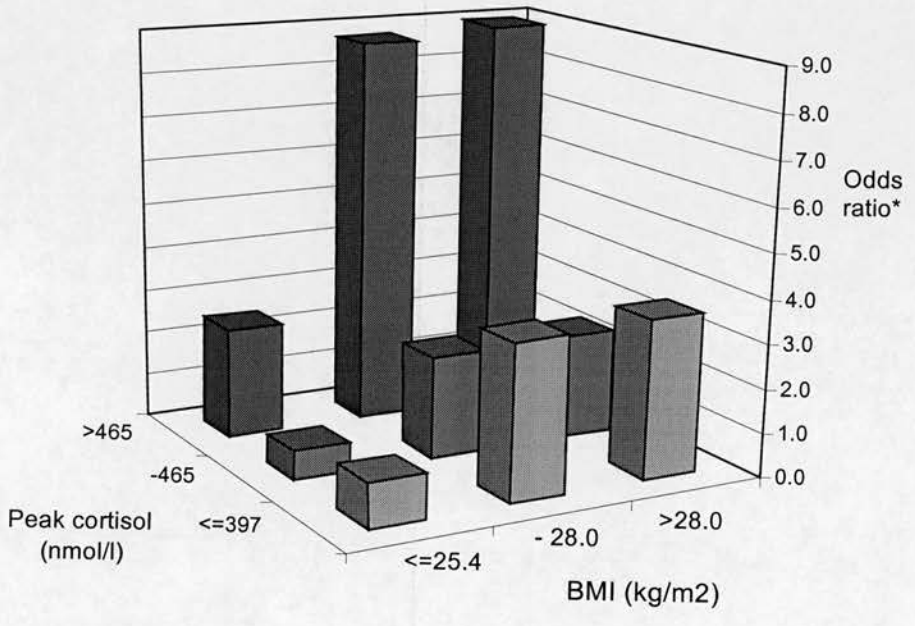
Table 3.2. Relationships between features of the Metabolic Syndrome and cortisol and its metabolites

		No. of men	*Plasma cortisol post-dexamethasone (nmol/l)* [a]	*Peak plasma cortisol post $ACTH_{1-24}$ (nmol/l)* [b]	*Cortisol profile*	*Total urinary cortisol metabolites (mg/24h)* [b]	*Total urinary cortisol metabolites per creatinine (mg/mmol)* [b]
Glucose tolerance	Normal	149	199.7 (91.0)	427.5 (1.2)		16.2 (2.0)	3.97 (1.97)
	IGT / Type 2 diabetes[c]	48	213.2 (101.5)	442.3 (1.2)		19.7 (2.3)	5.00 (2.34)
p-value			*ns*	*p=0.09*[d]	*p=0.05*[d]	*ns*	*ns*
				p=0.15[e]	*p=0.06*[e]		
Blood pressure	≤ 160mmHg	83	204.8 (101.2)	415.7 (1.2)		17.1 (1.8)	4.22 (1.78)
	> 160mmHg or treatment	120	200.5 (86.9)	442.2 (1.2)		17.0 (2.3)	4.17 (2.26)
p-value			*ns*	*p=0.02*[d]	*ns*	*ns*	*ns*
				p=0.03[e]			
Triglycerides	≤1.4mmol/l	102	196.8 (90.7)	418.0 (1.2)		15.6 (2.1)	3.82 (2.09)
	>1.4mmol/l	101	207.8 (95.0)	445.3 (1.2)		18.6 (2.0)	4.66 (1.54)
p-value			*ns*	*p=0.02*[d]	*p=0.08*[d]	*ns*	*ns*
				p=0.04[e]	*p=0.06*[e]		
Metabolic Syndrome[c]	absent	166	197.2 (90.5)	424.8 (1.2)		16.7 (2.0)	4.14 (1.99)
	present	31	234.3 (104.9)	466.4 (1.2)		18.4 (2.5)	4.53 (2.49)
p-value			*ns*	*p=0.01*[d]	*p=0.007*[d]	*ns*	*ns*
				p=0.04[e]	*p=0.006*[e]		
All		203	202.2 (92.8)	431.3 (1.2)		17.1 (2.1)	4.2 (7.8)

[a] arithmetic mean (sd)
[b] geometric mean (sd)
[c] defined in text
[d] *p* = *p*-value unadjusted
[e] *p* = *p*-value adjusted for BMI, social class and depression

Figure 3.3

Relative risk of the Metabolic Syndrome according to current body mass index (BMI) and the peak cortisol response to synacthen



* Relative to lowest peak and lowest BMI group

3.4 Discussion

The principal findings are that men with low birthweight and features of the Metabolic Syndrome have enhanced responsiveness of plasma cortisol to $ACTH_{1-24}$ and increased total urinary cortisol metabolite excretion, but normal plasma cortisol after dexamethasone. Indeed, plasma cortisol after $ACTH_{1-24}$ accounts for much of the effect of birthweight on features of the Metabolic Syndrome. These findings are consistent with the hypothesis that low birthweight is associated with increased activity of the HPA axis, and that this could contribute to the Metabolic Syndrome and attendant risk of cardiovascular disease.

By contrast with cortisol measurements in low birthweight men, these data show that obesity was associated with lower plasma cortisol following dexamethasone and no difference in responses to $ACTH_{1-24}$, in the face of increased urinary cortisol metabolite excretion, especially of 5α-reduced metabolites of cortisol. The lower plasma cortisol may be explained by increased peripheral metabolism of cortisol by 5α-reductases (Andrew *et al.* 1998). Indeed, increasing obesity and its associated increase in lean body mass (reflected in higher creatinine excretion) amongst high birthweight men confounded the relationship between birthweight and urinary cortisol metabolite excretion. This confounding effect resulted in a U-shaped unadjusted relationship between birthweight and cortisol metabolites, as described previously (Clark *et al.* 1996).

The explanation for activation of the HPA axis in men with lower birthweight and features of the adult Metabolic Syndrome remains unclear. Rats exposed to glucocorticoids *in utero* have increased plasma glucocorticoid levels and lower levels of GR in brain and pituitary gland, which may impair negative feedback control of CRH and ACTH secretion (Levitt *et al.* 1996). If the same 'programming' of GR expression occurred in man, then suppression of plasma cortisol by dexamethasone would be expected to be impaired in men with lower birthweight, but in this study it was preserved. Indeed, since fasting plasma cortisol is higher in low birthweight men without dexamethasone (Phillips *et al.* 1998; Phillips *et al.* 2000), but not

different post dexamethasone, the incremental effect of dexamethasone may be greater. However, in mice the entrance of low doses of synthetic glucocorticoids such as dexamethasone into the brain is blocked by a drug-exporting mdr1a encoding P-glycoprotein in the blood-brain barrier (Meijer *et al.* 1998). As mdr-1 is expressed in endothelial cells of human capillary blood vessels at the blood-brain barrier (Cordon-Cardo *et al.* 1989), P-glycoprotein could also regulate entrance of synthetic glucocorticoids into the central nervous system in man (Karssen *et al.* 2001). Therefore this low dose DST may only have tested the pituitary component of the negative feedback loop.

Another possibility is that increased cortisol secretion could result from increased adrenocortical sensitivity to ACTH. The measurements of other ACTH-dependent steroids exclude variance in cortisol response due to sub-clinical 21-hydroxylase deficiency (New 1998). Other corticosteroid biosynthetic defects which have been proposed as being important in hypertension, such as 11β-hydroxylase deficiency, predict lower rather than higher cortisol responses. Alternatively, the pattern of cortisol response to synacthen in low birthweight subjects with both an increased peak and a slower decline raises the possibility that they may have impaired plasma clearance of cortisol not revealed by 24 hour urinary cortisol analysis. Finally, elevated plasma cortisol may result from enhanced drive to CRH, ACTH and cortisol secretion from higher centres manifest as an increase in plasma cortisol when stressed on first sampling. Preliminary evidence supporting this proposal will be discussed in Chapter 4.

Chapter 4

Measurements of plasma cortisol during oral glucose tolerance tests: a preliminary study

4.1 Introduction

The mechanisms underlying the activation of the HPA axis in subjects with the Metabolic Syndrome (as in Chapter 3) are unknown. Obese subjects also have activation of the HPA axis (Marin *et al.* 1992; Pasquali *et al.* 1993; Andrew *et al.* 1998), but in contrast to the elevated plasma cortisol in subjects with the Metabolic Syndrome (Phillips *et al.* 1998; Phillips *et al.* 2000; Walker *et al.* 2000), plasma cortisol concentrations are low in obesity (Ljung *et al.* 1996; Walker *et al.* 2000). In obesity, increased peripheral clearance of cortisol may account for the compensatory activation of the HPA axis (Andrew *et al.* 1998). Alternatively, a number of metabolic responses to feeding may interact with the HPA axis, and be altered in the Metabolic Syndrome. It is known that plasma cortisol levels fall during an OGTT (Rodman and Bleicher 1973; Sober *et al.* 1977) and rise during a protein meal (Gibson *et al.* 1999). In the extreme, adrenal hypersensitivity to gastric inhibitory peptide can result in 'food-induced Cushing's syndrome' (Lebrethon *et al.* 1998). Therefore measurements of plasma cortisol during OGTTs may be a useful tool for revealing altered control of the HPA in the Metabolic Syndrome. However, it is not known whether the fall in plasma cortisol during the OGTT reflects the circadian fall in circulating hormone or whether ingestion of glucose affects the response. The aim was to determine this by performing the first placebo-controlled cross-over study of the effect of oral glucose on circulating plasma cortisol before measuring changes in plasma cortisol during OGTTs in a larger number of subjects (see Chapter 5). To further explore the associations between cortisol and glucose tolerance, both subjects with glucose intolerance and normoglycaemic controls were studied.

4.2 Methods

4.2a Clinical protocol

40 participants from the dexamethasone/$ACTH_{1-24}$ study were invited to take part in a further study of cortisol dynamics during OGTTs. These men were selected by their previous glucose tolerance data from 1991 with the aim of studying equal numbers with glucose intolerance and normal controls.

Following an overnight fast, subjects attended a clinic at Hertford County Hospital at 0830 h for OGTTs. A 21-gauge butterfly cannula was inserted in an antecubital fossa vein and after 30 min rest, a baseline blood sample was obtained. Subjects then drank either 75 g oral glucose (as 389 ml Traditional Lucozade Sparkling Glucose Drink) or placebo (identical in appearance and taste to Lucozade but containing no glucose, supplied by SmithKline Beecham). They returned a week later for a repeat test with the alternative solution in a single-blind crossover design. Venous blood was sampled from the cannula at 30, 60, 90 and 120 min for measurement of plasma glucose and insulin concentrations, following the glucose or placebo load. 29 subjects (17 glucose intolerant, 12 controls) received glucose in the first phase, while 10 subjects (5 glucose intolerant, 5 controls) received placebo first. Placebo and glucose phases were separated by at least one week.

4.2b Laboratory measurements

Blood samples were centrifuged, processed immediately and stored at -80°C for subsequent analysis. Plasma cortisol was measured by RIA as before (see 2.2c). Plasma glucose was measured by the hexokinase method and insulin by a two-site immunometric assay with either ^{125}I or alkaline phosphate labels.

4.2c Statistical analysis

As the distributions of cortisol measurements were skewed, $\log_e$ transformed variables were used in all analyses. Independent 2-sample t-tests were used to compare cortisol concentrations for control compared with glucose intolerant

subjects. ANOVA for repeated measures was used to analyse the plasma cortisol measurements during the glucose tolerance test. All analysis was conducted using Statistica 6.0 1997. Methods were checked by statistician HE Syddall, Southampton.

4.3 Results

One subject completed only one phase of the study and was therefore excluded from analysis. Using plasma glucose measurements following the 75 g oral glucose load, 22 subjects were defined as glucose intolerant (either IGT or Type 2 diabetes mellitus, defined as in 3.3.1), and 17 subjects as normal controls.

4.3.1 Differences in plasma cortisol between controls and glucose intolerant subjects without glucose

In all subjects plasma cortisol concentrations declined over the 120 min of the test following placebo. Glucose intolerant subjects had significantly higher cortisol concentrations following placebo than controls (p=0.001) (Figure 4.1). This difference was most marked at baseline and during the first 90 min of the test but was no longer present at 120 min. The differences in cortisol between glucose intolerant subjects and controls were not accounted for by variations in CBG.

4.3.2 Effect of treatment with glucose on plasma cortisol

Figure 4.2 shows that treatment with an oral glucose load blunted the circadian fall in plasma cortisol in both controls and glucose intolerant subjects (p=0.002). There was no significant difference in the effect of the glucose load in controls compared with those with glucose intolerance (controls p=0.02, glucose intolerant p=0.04, interaction p=0.50).

4.3.3 Order of test: habituation to venepuncture

0900 h plasma cortisol concentrations were significantly higher in the first phase of the study than in the second phase in controls (p=0.01) (Figure 4.3). However, in glucose intolerant subjects, 0900 h plasma cortisol concentrations in the two study

Figure 4.1 **Differences in plasma cortisol between controls and glucose intolerant subjects following placebo**

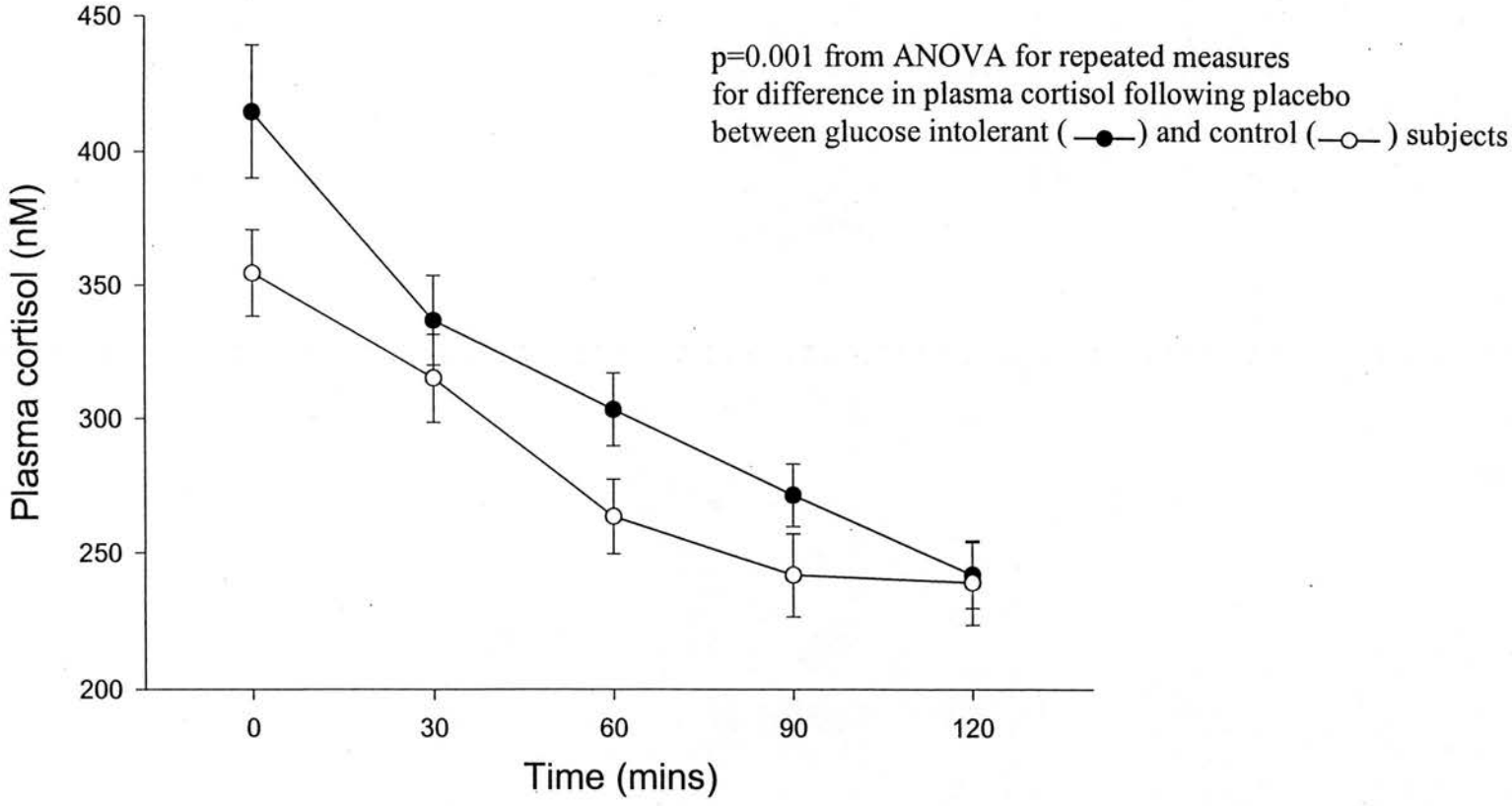
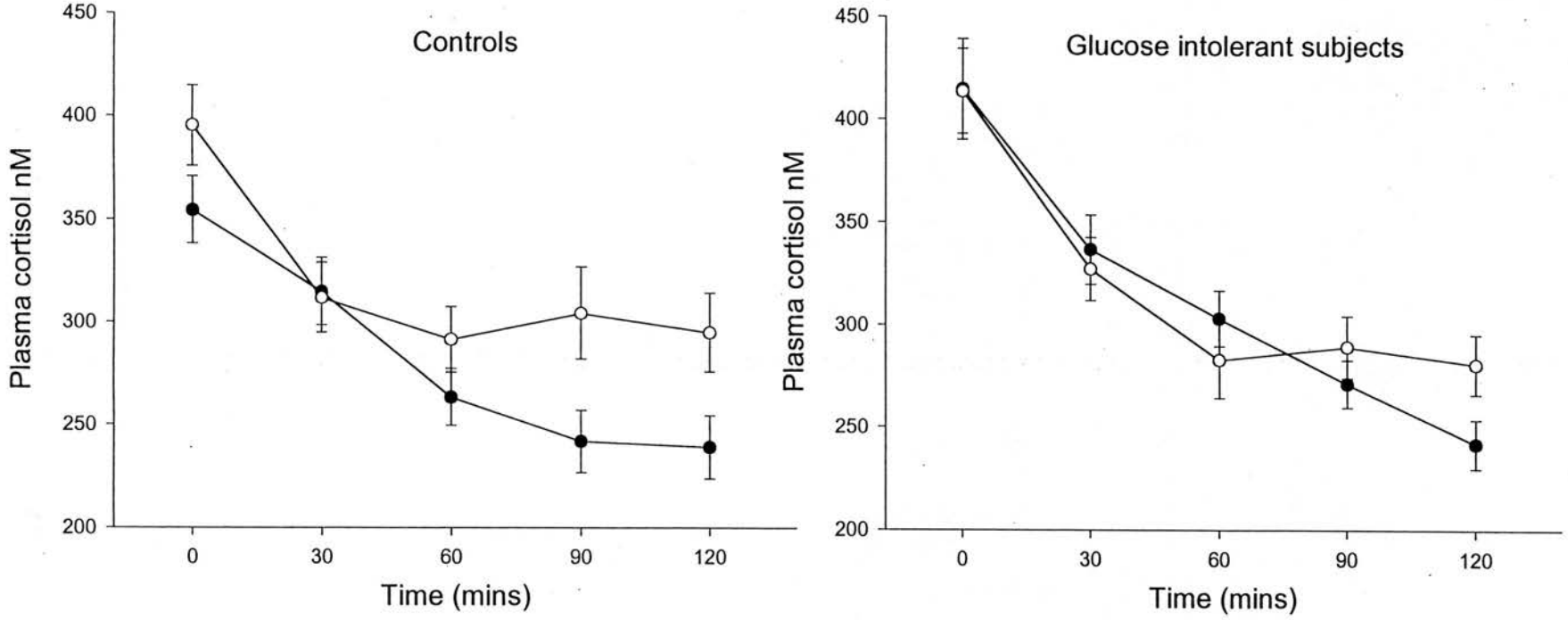


Figure 4.2 Effect of glucose on plasma cortisol



plasma cortisol following placebo
plasma cortisol following glucose

p=0.002 from ANOVA for repeated measures for effect of glucose in raising plasma cortisol in all subjects.
No significant difference in effect of glucose between controls and glucose intolerant subjects (p=0.02 for controls, p=0.04 for glucose intolerant subjects)

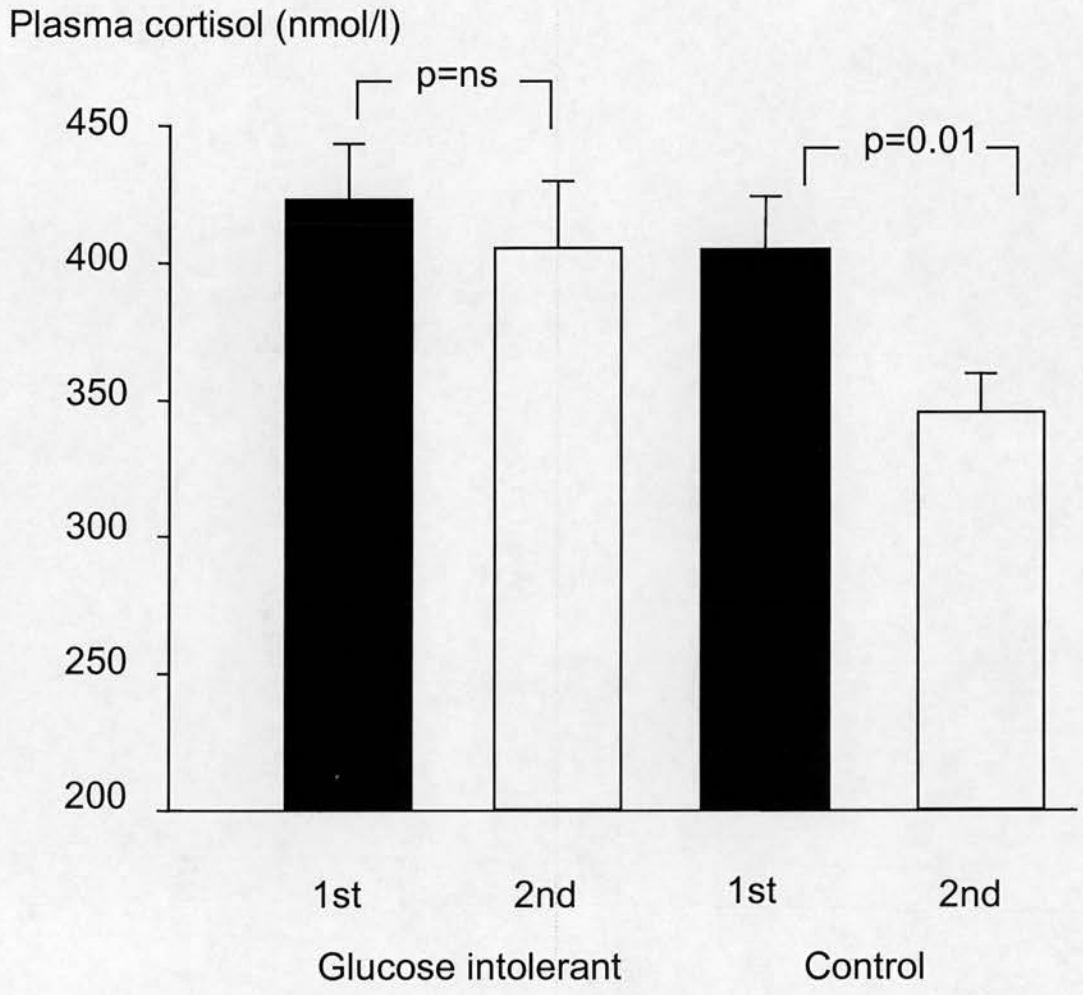


Figure 4.3

Effect of order of study phase on 0900 h plasma cortisol in glucose intolerant and control subjects

First phase, *black columns*; second phase, *white columns*
p = 0.01 for effect of order in control subjects; no significant order effect in glucose intolerant subjects.

phases were not different (p=0.18) as this measurement did not fall in the second study phase.

4.3.4 Effect of treatment with glucose and order of test on plasma glucose and insulin concentrations

Both plasma glucose and insulin concentrations were higher after treatment with glucose than placebo ($p<0.001$) and were significantly higher in glucose intolerant subjects than controls. In contrast to the plasma cortisol concentrations, plasma glucose and insulin concentrations were no different in the two study phases in either glucose intolerant subjects or controls.

4.4 Discussion

Treatment with an oral glucose load blunted the normal circadian fall in plasma cortisol in both subjects with glucose intolerance and in normoglycaemic controls. However, subjects with glucose intolerance had higher cortisol concentrations following placebo, suggesting that their normal diurnal fall in plasma cortisol differed from controls. Baseline plasma cortisol concentrations were higher during the first phase of the study in control subjects suggesting a stress response associated with the novelty of first clinic attendance. In addition, subjects with glucose intolerance had high plasma cortisol concentrations at the second clinic attendance suggesting a lack of habituation to repeated measurement of plasma cortisol.

It is not known whether the fall in cortisol during an OGTT reflects the circadian fall in plasma cortisol or whether glucose ingestion affects the response. These studies were performed in the morning when any alterations in circadian rhythm should be most clearly demonstrated. Indeed, subjects with glucose intolerance had higher plasma cortisol than controls during the first 90 min of the placebo phase of the study. Altered diurnal rhythms of salivary cortisol in subjects with glucose intolerance have been reported (Rosmond *et al.* 1998), and this finding is consistent with the associations between 0900 h plasma cortisol and glucose intolerance (Phillips *et al.* 1998; Phillips *et al.* 2000).

Despite the differences in circadian fall of plasma cortisol, the effect of glucose ingestion was to raise plasma cortisol in both normal and glucose intolerant subjects. Plasma and salivary cortisol concentrations rise following a meal (Rosmond *et al.* 1998), particularly if the meal is of high protein content (Gibson *et al.* 1999), as protein ingestion stimulates pituitary ACTH secretion (Al-Damluji *et al.* 1987). Although it has previously been suggested that carbohydrate ingestion has little effect on the HPA axis (Ishizuka *et al.* 1983), this study now demonstrates that an oral glucose load raises plasma cortisol. It remains unknown whether this is a consequence of the glucose load itself, or whether the associated insulin release affects cortisol metabolism. For example, insulin may modulate adrenal steroidogenesis by inhibiting 17,20-lyase thereby favouring cortisol synthesis in preference to dehydroepiandrosterone and androstenedione (Nestler *et al.* 1992). Insulin may also affect cortisol metabolism by decreasing the activity of 11β-HSD1 which acts primarily as a reductase mediating conversion of inactive cortisone to active cortisol (Jamieson *et al.* 1995). Insulin may also act centrally on the HPA axis regulating drive, although results of studies to date are conflicting, either showing a decreased cortisol response to CRH following insulin infusion (Walker *et al.* 1994), or increased ACTH secretion following supraphysiological hyperinsulinaemia (Fruehwald-Shultes *et al.* 1999). However, as the effect of treatment with the glucose load did not differ between glucose intolerant subjects and controls, it would appear less likely that the hyperglycaemia or changes in insulin concentrations associated with glucose intolerance influenced the fall in cortisol concentrations during the test.

An alternative hypothesis to explain the elevated plasma cortisol during the glucose tolerance test is a response to stress. To reduce any effect of stress the study was conducted the study in familiar surroundings, with staff previously known to the subjects. Yet fasting plasma cortisol measured at the first phase attendance, and so arguably the most stressful visit, was significantly higher than that at the second phase in control subjects. Elevated plasma and salivary cortisol concentrations are

observed in situations of increased perceived stress (Rosmond *et al.* 1998) and the stress of venepuncture is known to raise plasma cortisol (Meeran *et al.* 1993). The effect of psychosocial stress on raising cortisol has also been reported to be greater after a glucose load (Kirschbaum *et al.* 1997).

Most interestingly, glucose intolerant subjects also had high baseline plasma cortisol concentrations in the second phase of the study, indicating a lack of habituation to repeated stress. A similar lack of habituation of blood pressure and heart rate responses to repeated restraint stress is seen in spontaneously hypertensive rats compared with normal controls (McDougall *et al.* 2000). As discussed in Chapter 3, subjects with glucose intolerance and /or low birthweight have evidence of activation of the HPA axis, which was not accounted for by differences in central feedback sensitivity. The elevated plasma cortisol from stress of repeated venous sampling seen in this study would be consistent with the hypothesis that the activation of the HPA axis is due to enhanced drive to CRH, ACTH and cortisol secretion from higher centres.

Chapter 5

Predicting cardiovascular risk factors from plasma cortisol measured during oral glucose tolerance tests

5.1 Introduction

The findings in Chapter 3 show that there is activation of the HPA axis in the Metabolic Syndrome, although the underlying mechanisms remain unclear. The data also show that activation of the HPA axis is predicted by low birthweight, supporting the hypothesis that it may result from permanent alteration in the central control of the HPA axis as a result of events in early life (Seckl 1998). Indeed the findings in Chapter 4 are consistent with increased central drive to cortisol secretion. However, in Chapter 4, plasma cortisol following an oral glucose load did not differ in subjects with glucose intolerance compared with normoglycaemic controls. Also, in another study plasma cortisol measured 2 hours following an oral glucose load was less predictive of cardiovascular risk than the fasting measurement (Walker *et al.* 2000), suggesting that the fall in cortisol might be less in subjects with the Metabolic Syndrome.

Most of the published cross-sectional studies relating cortisol levels to cardiovascular risk factors have been performed in men, whereas most of the case-control studies of cortisol in obese subjects have included women. Therefore it is not known whether the associations of cortisol with the Metabolic Syndrome are the same in women and the extent to which these relationships are confounded by obesity. The aim therefore was to measure plasma cortisol during OGTTs in a larger number of both men and women to determine correlates of changes in plasma cortisol with features of the Metabolic Syndrome and to examine the extent to which these relationships are confounded by obesity.

5.2 Methods

5.2a Subjects

The Hertfordshire cohort has been previously described (Chapter 3.2a). 370 of these men underwent 75-g OGTTs in 1991. Measurements of blood pressure and height, weight, waist and hip circumferences were recorded (Hales *et al.* 1991). The other subjects were participants from another epidemiological study conducted by the MRC Environmental Epidemiology Unit in Southampton. Between 1935 and 1943, a standardised record form was kept for each woman admitted to the Sharoe Green Hospital in Preston, Lancashire, England. This record included the date of the mother's last menstrual period and the baby's birthweight, placental weight (both recorded in pounds), and length from crown to heel and head circumference (both recorded in inches). The National Health Service central register was used to trace 503 people born in the hospital between 1935 and 1943, and still living in Lancashire. In 1993, 266 men and women living in or close to Preston attended Sharoe Green Hospital for a 75-g OGTT. Anthropometric measurements and blood pressure were recorded (Phipps *et al.* 1993).

5.2b Laboratory measurements

Plasma cortisol was assayed by RIA in the fasting, 30 min and 120 min samples from the OGTT (see 2.2c). There was sufficient plasma for complete cortisol results from 339 men in the Hertfordshire cohort and 254 subjects (135 men, 119 women) in the Preston cohort. None of the subjects had pituitary or adrenal disease and two subjects on oral prednisolone treatment were excluded from the Preston analysis.

5.2c Statistical analysis

The area under the cortisol curve (cortisol AUC) was used as a summary measurement of plasma cortisol during the OGTT by calculating the area under the trapezium described by the cortisol measurements at baseline, 30 min and 120 min (units h.nmol/l). Likewise the areas under the glucose (glucose AUC) and insulin (insulin AUC) curves during the OGTT were calculated. In addition, the percentage decline in cortisol from baseline to 120 min during the OGTT was calculated as 100*(($\log_e$cortisol 0 min-$\log_e$cortisol 120 min) / $\log_e$cortisol 0 min). The insulin

increment, a previously validated index of insulin secretion (Phillips *et al.* 1994b), was calculated from the plasma measurements during the OGTT as $\log_e$((insulin 30 min – insulin 0 min)/(glucose 30 min – glucose 0 min)). The distributions of measurements of blood pressure, triglycerides, cholesterol, cortisol, glucose, insulin and AUCs were $\log_e$-transformed and geometric means and sds are presented. As the distribution of BMI was positively skewed in the Preston data set but was not satisfactorily transformed by the $\log_e$ transformation, Fisher-Yates normal-scores for BMI were used as a measure of obesity. Pearson correlation coefficients were computed to examine the pair-wise associations between variables. Associations between cortisol, BMI and glucose and insulin measurements during the OGTT were investigated by multiple linear regression. All statistical analysis was conducted by statistician HE Syddall, Southampton.

5.3 Results

5.3.1 Subject characteristics

Table 5.1 shows the characteristics of the participants and the measurements of plasma glucose, insulin and cortisol during the OGTT. The Hertfordshire men were older but of similar BMI to the Preston men. The Preston men and women were of similar ages but the men were of higher BMI ($p=0.01$) and WHR ($p<0.0001$). The Preston men had higher glucose AUC ($p<0.001$ unadjusted, $p=0.002$ adjusted for BMI) and tended to have higher insulin AUC during the OGTT (p=ns) than the women. Blood pressure was higher in men than in women.

Table 5.1 Characteristics of participants

Mean (sd)[a]	*Hertfordshire men (n=339)*	*Preston men (n=135)*	*Preston women (n=119)*
Age (y)	64.6 (3.2)	51.8 (2.2)	51.3 (2.4)
BMI (kg/m^2)	26.9 (3.6)	25.7 (3.4)	24.7 (4.4)
WHR	0.9 (0.1)	0.9 (0.1)	0.8 (0.1)
Number with IGT/Type 2 DM	n=84	n=14	n=20
Plasma glucose (mmol/l)			
Fasting	6.1 (1.2)	5.8 (1.1)	5.4 (1.1)
30 min	9.4 (1.2)	9.0 (1.3)	7.5 (1.3)
120 min	6.6 (1.4)	5.5 (1.3)	5.8 (1.3)
Glucose AUC (h.mmol/l)	16.0 (1.3)	14.7 (1.2)	13.4 (1.3)
Plasma insulin (pmol/l)			
Fasting	43 (1.9)	45 (1.7)	42 (1.6)
30 min	275 (1.9)	288 (1.7)	249 (1.7)
120 min	150 (2.4)	147 (2.2)	178 (2.0)
Insulin AUC (h.pmol/l)	425 (1.8)	439 (1.6)	420 (1.6)
Insulin increment (pmol/mmol)	70.5 (2.3)	81.4 (2.2)	94.2 (2.1)
Plasma cortisol (nmol/l)			
Fasting	320 (1.4)	427 (1.3)	403 (1.4)
30 min	349 (1.5)	425 (1.5)	362 (1.4)
120 min	259 (1.4)	276 (1.4)	245 (1.4)
Cortisol AUC (h.nmol/l)	634 (1.4)	749 (1.4)	656 (1.3)
Systolic blood pressure (mmHg)	164 (1.2)	138 (1.2)	130 (1.2)
Diastolic blood pressure (mmHg)	90 (1.1)	78 (1.1)	73 (1.2)
Triglycerides (mmol/l)	1.4 (1.7)	1.4 (1.7)	1.1 (1.6)
HDL cholesterol (mmol/l)	1.2 (1.3)	1.2 (1.3)	1.5 (1.3)
LDL cholesterol (mmol/l)	4.7 (1.3)	4.4 (1.3)	4.3 (1.3)

[a]Values are geometric mean (sd) for all variables except age and WHR for which arithmetic means (sd) are given, and BMI for which the arithmetic mean (sd) is given for Hertfordshire, and the median (and inter-quartile range) is given for Preston.

5.3.2 Plasma cortisol concentrations during the OGTT

In all subjects plasma cortisol concentrations fell during the OGTT ($p<0.0001$). Table 5.2a shows the associations between cortisol AUC and clinical features of the metabolic syndrome. In men and women, higher cortisol AUC was associated with higher glucose AUC and higher systolic blood pressure. Higher cortisol AUC was associated with reduced insulin increment in men, but higher 2 h insulin and insulin AUC in women. Table 5.2b shows the associations between the change in plasma cortisol following the oral glucose load and clinical features of the metabolic syndrome. The only statistically significant associations were in the Preston men where a smaller decline in plasma cortisol was associated with higher 2 h glucose and glucose AUC, lower systolic blood pressure, higher HDL cholesterol and reduced insulin increment.

5.3.3 Obesity

Obesity, as measured by BMI, was associated with both lower fasting cortisol (Hertfordshire $p=0.02$; Preston $p=0.04$ unadjusted, $p=0.02$ adjusted for gender) and a lower cortisol AUC (Hertfordshire $p=0.0001$; Preston $p=0.05$ unadjusted, $p=0.01$ adjusted for gender), but not with the percentage decline in cortisol. Tables 5.2a and 5.2b show that after adjustment for the confounding effect of obesity, the associations between cortisol AUC or percentage decline in plasma cortisol and clinical features of the metabolic syndrome were strengthened.

Table 5.2a **Associations between cortisol AUC during OGTT and features of the Metabolic Syndrome in 339 Hertfordshire men, 135 Preston men and 119 Preston women**

	Hertfordshire men					*Preston men*					*Preston women*				
	n	*%change (95% CI) in Cortisol AUC per geometric sd increase in feature*		*p-value*	*[a]p-value*	*[b]n*	*%change (95% CI) in Cortisol AUC per geometric sd increase in feature*		*p-value*	*[a]p-value*	*[b]n*	*%change (95% CI) in Cortisol AUC per geometric sd increase in feature*		*p-value*	*[a]p-value*
2 h glucose (mmol/l)	339	2.4	(-0.8,5.8)	0.14	0.03	135	6.0	(0.6, 11.7)	0.03	0.002	119	1.3	(-3.7, 6.6)	0.61	0.36
glucose AUC (h.mmol)	339	3.8	(0.5, 7.2)	0.02	0.001	135	7.5	(2.1, 13.2)	0.006	<0.001	119	6.2	(1.1, 11.6)	0.02	0.01
2 h insulin (pmol/l)	339	-1.3	(-4.5, 1.9)	0.41	0.90	132	-0.0	(-5.2, 5.3)	0.96	0.73	116	6.1	(0.9, 11.6)	0.02	0.04
insulin AUC (h.pmol)	339	-2.5	(-5.5, 0.7)	0.13	0.69	125	1.5	(-3.9, 7.3)	0.58	0.41	111	8.6	(3.4, 14.0)	0.001	0.001
insulin increment (pmol/mmol)	339	-5.5	(-8.5, -2.5)	0.001	0.001	126	-6.2	(-11.2, 0.9)	0.02	0.01	104	-0.4	(-5.7, 5.2)	0.89	0.95
Systolic BP (mmHg)	339	4.6	(1.3, 8.0)	0.006	<0.001	135	3.2	(-2.1, 8.7)	0.25	0.05	119	5.7	(0.6, 11.2)	0.03	0.02
Diastolic BP (mmHg)	339	4.3	(1.0, 7.6)	0.01	<0.001	135	4.6	(-0.8, 10.2)	0.09	0.02	119	0.2	(-4.7, 5.5)	0.93	0.76
Triglycerides (mmol/l)	338	-0.0	(-3.2, 3.2)	0.98	0.22	135	2.0	(-3.2, 7.6)	0.45	0.19	119	2.8	(-2.3, 8.1)	0.29	0.14
HDL Cholesterol (mmol/l)	327	3.1	(-0.0, 6.5)	0.06	0.13	132	7.1	(1.6, 12.9)	0.01	0.04	116	1.3	(-3.8, 6.7)	0.63	0.97
LDL Cholesterol (mmol/l)	338	1.9	(-1.3, 5.2)	0.25	0.11	132	2.2	(-3.2, 7.9)	0.43	0.29	116	-0.3	(-5.4, 5.0)	0.92	0.98

[a] p-value for % change in Cortisol AUC adjusted for BMI
[b] 5 Preston men and 3 Preston women had missing values for BMI

Table 5.2b **Associations between percentage decline in cortisol between 0 min and 120 min during OGTT and features of the Metabolic Syndrome in 339 Hertfordshire men, 135 Preston men and 119 Preston women**

	Hertfordshire men				*Preston men*				*Preston women*			
	n	*change (95% CI) in percentage decline of cortisol per geometric sd increase in feature*	*p-value*	*[a]p-value*	*n*	*change (95% CI) in percentage decline of cortisol per geometric sd increase in feature*	*p-value*	*[a]p-value*	*n*	*change (95% CI) in percentage decline of cortisol per geometric sd increase in feature*	*p-value*	*[a]p-value*
2 h glucose (mmol/l)	339	-0.24 (-0.93, 0.44)	0.49	0.44	135	-1.22 (-2.23, -0.22)	0.02	0.03	119	-0.65 (-1.80, 0.49)	0.26	0.18
glucose AUC (h.mmol)	339	0.02 (-0.67, 0.71)	0.95	0.97	135	-0.96 (-1.97, 0.05)	0.06	0.22	119	-0.75 (-1.89, 0.39)	0.20	0.16
2 h insulin (pmol/l)	339	-0.05 (-0.73, 0.64)	0.90	0.81	132	0.13 (-0.89, 1.15)	0.80	0.67	116	-0.43 (-1.60, 0.75)	0.48	0.47
insulin AUC (h.pmol)	339	0.36 (-0.33, 1.05)	0.30	0.35	125	0.26 (-0.81, 1.33)	0.63	0.51	111	0.10 (-1.03, 1.23)	0.86	0.82
insulin increment (pmol/mmol)	339	0.32 (-0.37, 1.01)	0.37	0.38	126	1.13 (0.06, 2.20)	0.04	0.12	104	1.19 (0.04, 2.34)	0.04	0.03
Systolic BP (mmHg)	339	-0.21 (-0.90, 0.47)	0.54	0.47	135	1.07 (0.07, 2.08)	0.04	0.01	119	-0.19 (-1.34, 0.96)	0.75	0.76
Diastolic BP (mmHg)	339	-0.21 (-0.90, 0.47)	0.54	0.48	135	0.51 (-0.51, 1.53)	0.32	0.26	119	-0.78 (-1.93, 0.36)	0.18	0.16
Triglycerides (mmol/l)	338	0.38 (-0.31, 1.07)	0.28	0.32	135	0.43 (-0.59, 1.45)	0.40	0.22	119	0.88 (-0.26, 2.03)	0.13	0.16
HDL Cholesterol (mmol/l)	327	-0.33 (-1.02, 0.36)	0.35	0.37	132	-1.76 (-2.76, -0.76)	0.001	<0.001	116	-0.22 (-1.41, 0.96)	0.71	0.89
LDL Cholesterol (mmol/l)	338	-0.14 (-0.83, 0.55)	0.68	0.65	132	-0.49 (-1.53, 0.55)	0.36	0.32	116	0.37 (-0.81, 1.55)	0.53	0.58

[a] p-value for change in percentage decline in cortisol adjusted for BMI
[b] 5 Preston men and 3 Preston women had missing values for BMI

5.3.4 Independent effects of plasma cortisol and obesity on plasma glucose

The independent effects of plasma cortisol and obesity on plasma glucose are illustrated in Tables 5.3a and 5.3b. In both cohorts at any BMI, higher cortisol AUC was associated with higher glucose AUC, while at any cortisol AUC, higher BMI was associated with higher glucose AUC. Thus, the highest glucose AUC was observed in subjects with highest cortisol AUC and highest BMI, and the lowest in those with lowest cortisol AUC and least BMI. In a multiple regression analysis with glucose AUC as the dependent variable and cortisol AUC, BMI, age and gender as independent variables, cortisol AUC ($p=0.001$), BMI ($p<0.001$), and age ($p=0.01$) were all significant predictors of glucose AUC in Hertfordshire, and cortisol AUC ($p<0.001$), BMI ($p<0.001$) and gender ($p=0.03$, women with lower glucose AUC than men) were strongest predictors of glucose AUC in Preston. There was no significant interaction between cortisol AUC and obesity in either cohort (Hertfordshire $p=0.99$, Preston $p=0.73$). The association between glucose AUC and both cortisol AUC and obesity was also observed after exclusion of the 84 Hertfordshire and 34 Preston subjects with IGT or Type 2 diabetes mellitus, although the strengths of the associations were reduced.

Table 5.3a Effects of cortisol AUC and BMI on glucose AUC in Hertfordshire

Hertfordshire Men Mean[a] (n)		*Tertiles of BMI (kg/m²)[c]*			
		Lowest	*Middle*	*Highest*	*All*
Tertiles of Cortisol AUC (h.nmol/l)[b]	*Lowest*	13.8 *(n=27)*	15.6 *(n=42)*	16.3 *(n=44)*	15.4 *(n=113)*
	Middle	15.2 *(n=42)*	15.5 *(n=36)*	17.0 *(n=36)*	15.9 *(n=114)*
	Highest	16.2 *(n=44)*	16.2 *(n=36)*	18.7 *(n=32)*	16.9 *(n=112)*
	All	15.3 *(n=113)*	15.8 *(n=114)*	17.2 *(n=112)*	16.0 *(n=339)*

[a] Geometric mean of glucose AUC. The geometric sd ranged between 1.2 and 1.4
[b] Tertiles of cortisol AUC (h.nmol/l): ≤ 552, -720, >720
[c] Tertiles of BMI (kg/m²): < 25.5, -28.0, ≥ 28.0

Table 5.3b Effects of cortisol AUC and BMI on glucose AUC in Preston

Preston Men and Women Mean[a, b] (n)		*Tertiles of BMI (kg/m²)[d]*			
		Lowest	*Middle*	*Highest*	*All*
Tertiles of Cortisol AUC (h.nmol/l)[c]	*Lowest*	12.8 *(n=27)*	13.5 *(n=29)*	14.1 *(n=25)*	13.4 *(n=81)*
	Middle	13.5 *(n=33)*	13.7 *(n=22)*	14.2 *(n=27)*	13.8 *(n=82)*
	Highest	14.2 *(n=40)*	14.5 *(n=25)*	16.9 *(n=18)*	14.8 *(n=83)*
	All	13.6 *(n=100)*	13.9 *(n=76)*	14.8 *(n=70)*	14.0 *(n=246)*

[a] Geometric mean of glucose AUC. The geometric sd ranged between 1.1 and 1.3
[b] Results in Table 5.3b combined for men and women
Effect is the same if analysed separately but results for women are weaker
[c] Tertiles of cortisol AUC (h.nmol/l):
Preston men ≤ 635, -841, >841
Preston women ≤ 573, -740, >740
[d] Tertiles of BMI (kg/m²):
Preston men ≤ 27, -27, > 27
Preston women ≤ 24, -26, > 26

5.3.5 Association of plasma cortisol and birthweight

Table 5.4 shows the associations between cortisol AUC and birthweight. In the Hertfordshire men low birthweight was associated with higher cortisol AUC (p=0.006 unadjusted, p=0.03 adjusted for BMI). In the Preston cohort low birthweight was associated with higher cortisol AUC (p=0.03 adjusted for gender, p=0.04 adjusted for gender and BMI). The association was stronger in women than men but there was no statistically significant interaction between gender and birthweight as predictors of cortisol AUC (p=0.44 for interaction). Addition of birthweight into the above multiple regression model revealed low birthweight was also a significant predictor of high glucose AUC and did not reduce the strength of effects of the other predictors (cortisol AUC p<0.001, BMI p<0.001, gender p=0.02, birthweight p=0.002). There were no significant interactions between birthweight and cortisol AUC or birthweight and BMI. Birthweight was not associated with percentage decline in cortisol following the glucose load.

Table 5.4 Associations of cortisol AUC (h.nmol/l) during OGTT[a] and birthweight

	Hertfordshire men		*Preston men*		*Preston women*	
Birthweight lb	*n*	*Cortisol AUC*	*n*	*Cortisol AUC*	*n*	*Cortisol AUC*
- 6.5	56	682 (1.4)	44	785 (1.4)	38	713 (1.3)
-8.5	209	641 (1.4)	49	716 (1.3)	46	627 (1.4)
>8.5	74	581 (1.4)	42	751 (1.4)	35	636 (1.3)
All	339	634 (1.4)	135	749 (1.4)	119	656
		[b]p=0.006		*[d]p=0.28*		*[d]p=0.03*
		[c]p=0.03		*[e]p=0.33*		*[e]p=0.03*
						[d,f]p=0.03
						[e,f]p=0.04

[a] Values are geometric means and standard deviations
p-values from regression analyses of cortisol AUC in birthweight
[b] unadjusted
[c] adjusted for BMI
[d] adjusted for gender
[e] adjusted for gender and BMI
[f] Preston men and women together, no significant interaction between gender and birthweight, p=0.44

5.4 Discussion

In both of these populations in whom raised fasting plasma cortisol is associated with clinical features of the Metabolic Syndrome (Phillips *et al.* 1998; Phillips *et al.* 2000), high cortisol AUC during the OGTT also predicts cardiovascular risk. Likewise, in addition to the previously described association between low birthweight and elevated fasting plasma cortisol, low birthweight was associated with higher cortisol AUC.

The associations are sex-specific: higher cortisol AUC was associated with higher systolic blood pressure in both sexes. In women, high cortisol AUC was also associated with high glucose and insulin AUCs. However, in men high cortisol AUC was associated with high glucose AUC but not with hyperinsulinaemia, suggesting insulin deficiency rather than insulin resistance. Consistent with this, high cortisol AUC was associated with reduced insulin increment, a good correlate of first phase insulin secretion (Phillips *et al.* 1994b), which is interesting as glucocorticoids can directly inhibit insulin release from pancreatic β-cells (Delauney *et al.* 1997).

However, the decline in plasma cortisol after glucose administration was poorly predictive of features of the Metabolic Syndrome, being weakly significant only in the Preston men. This may not be surprising in light of the findings of Chapter 4, and in other studies plasma cortisol measured 2 hours following a glucose load has been less predictive of cardiovascular risk than the fasting measurement (Walker *et al.* 2000). The fall in plasma cortisol following an oral glucose load is influenced by the diurnal fall in cortisol secretion, which is altered in glucose intolerant subjects (Rosmond *et al.* 1998; Chapter 4), and also by the effect of oral glucose to raise plasma cortisol (Chapter 4).

This study also confirms the previously reported contrasting effects of obesity and cortisol on glucose intolerance (Walker *et al.* 2000). Several studies have shown that regulation of the HPA axis is altered in obesity (Marin *et al.* 1992; Pasquali *et al.* 1993; Andrew *et al.* 1998), notably that obesity is associated with lower plasma cortisol concentrations (Ljung *et al.* 1996) and increased peripheral clearance of

cortisol (Strain *et al.* 1980; Andrew *et al.* 1998). Consistent with the hypothesis that elevated plasma cortisol and obesity represent different mechanistic pathways to cardiovascular risk, the effect of BMI and plasma cortisol in this study, as in a previous study (Walker *et al.* 2000) were independent and additive. Thus, increasing BMI strengthened the associations between cortisol and glucose intolerance such that the highest glucose AUC was observed in subjects with the combination of obesity and high plasma cortisol concentrations.

The OGTT is commonly used in epidemiological studies of cardiovascular risk and also provides a dynamic test of the HPA axis since oral glucose raises plasma cortisol levels. However, although measurement of plasma cortisol during the glucose tolerance test reinforces the interpretation of the relationships between fasting plasma cortisol and cardiovascular risk factors (Phillips *et al.* 1998; Phillips *et al.* 2000), it did not offer additional value over measurement of fasting cortisol. While subtle differences in diurnal variation of plasma cortisol and in response to oral glucose might have obscured each other, it seems unlikely that altered HPA response to feeding is primarily responsible for HPA activation in subjects with the Metabolic Syndrome.

Chapter 6

Measuring peripheral glucocorticoid receptor expression in skeletal muscle from men with cardiovascular risk factors

6.1 Introduction

The findings from the clinical studies are consistent with the hypothesis that activation of the HPA axis (Chapter 3), with increased drive to cortisol secretion from higher centres (Chapter 4) leads to differences in insulin sensitivity and cardiovascular risk factors in the population. An alternative mechanism leading to variations in cortisol action is of altered tissue responsiveness to glucocorticoids. Tissue sensitivity to cortisol is largely determined by the density of GR expression. In man, the dermal vasoconstrictor response to topical steroids, an indirect measure of tissue sensitivity to cortisol, is increased in men with glucose intolerance and insulin resistance (Walker *et al.* 1998b), and also in men with essential hypertension (Walker *et al.* 1996), or with a familial predisposition to hypertension (Walker *et al.* 1998a). This response is also enhanced in healthy subjects who carry a polymorphism within a non-coding part of the GR gene (Panarelli *et al.* 1998) which is associated with a familial predisposition to hypertension (Watt *et al.* 1992) and with greater hyperinsulinaemia in obese subjects (Weaver *et al.* 1992).

Little is known about GR expression in man *in vivo*, partly due to problems of tissue accessibility. Skeletal muscle is a major determinant of insulin-stimulated glucose disposal and a primary site of insulin resistance, and moreover is a suitable tissue to biopsy. A collaboration with Professor Paul McKeigue, London School of Hygiene & Tropical Medicine, Keppel Street, London, WC1E 7HT, allowed access to a valuable resource of skeletal muscle biopsies from men from the Uppsala Longitudinal Study of Adult Men (McKeigue *et al.* 1998). The aim was to examine whether there was a relationship between skeletal muscle GR expression and features of the Metabolic Syndrome. In order to determine changes in GR mRNA levels in small sample sizes, a quantitative competitive reverse transcriptase (RT)-PCR assay for measuring human GR mRNA levels was developed.

6.2 Methods

6.2a Subjects

From the Uppsala Longitudinal Study of Adult Men (McKeigue *et al.* 1998), a sample of 99 men were selected who had been studied at age 70 and for whom muscle biopsy specimens were available. These men were part of a cohort of men born between 1920 and 1924 and living in Uppsala, Sweden, whose birthweights were known, and who had participated in a health investigation from 1970 to 1973. At a follow-up study in 1991, when the men were aged 70 years they underwent a 75g OGTT and euglycaemic hyperinsulinaemic clamp. Height, weight, waist and hip circumferences were measured, and 24 h ambulatory blood pressures recorded. On a separate day they attended after an overnight fast for a muscle biopsy. A biopsy of approximately 100 mg of muscle was taken from the vastus lateralis muscle via an 8 mm incision through the skin and fascia, using a Bergstrom needle under local anaesthesia. Biopsies were divided in half, snap frozen in liquid nitrogen and stored at -80°C. One half of each sample was used for RNA preparation. GR mRNA levels were determined using the competitive quantitative RT-PCR assay described below.

6.2b Competitive quantitative RT-PCR assay

Competitive quantitative RT-PCR is the most sensitive technique for measuring mRNA copy numbers (Clementi *et al.* 1993). Although there are several different methods for competitive RT-PCR, use of a synthetic RNA competitor, especially one possessing sequences nearly identical to the target RNA, controls for the efficiencies of both reverse transcription and amplification.

b(i) Tissues

Total RNA was extracted from vastus lateralis skeletal muscle biopsies using the TRIzol method (Appendix A 3.2). Integrity of RNA was verified by denaturing agarose gel electrophoresis (Appendix A 3.1b). Of the 99 available samples, RNA suitable for use in the assay was made from 24 samples. 23 samples were too small to yield RNA (either because samples were too small to yield RNA or the muscle

was attached to connective tissue or fat), and in 52 the extracted RNA was degraded and RT-PCR did not yield detectable bands for GR and 18S.

b(ii) Competitors

RNA competitors for human GR mRNA and human 18S mRNA (used as an internal control for variability in target RNA) were synthesised separately but under the same conditions (Benchmarks 1994, Appendix A 3.4.1(i)). Both competitors contain 83 base pair (bp) deletions to distinguish the PCR products derived from endogenous and synthetic RNAs.

b(iii) Assay

Competitive quantitative RT-PCR was carried out using 100 ng total target RNA in the assay (Appendix A 3.4.1(ii)). Because of small sample size, the competitive quantitative RT-PCR was performed once for each sample and only repeated if the competitive reaction was unsatisfactory (correlation less than 0.9). The inter-assay coefficient of variation was 12%. As an additional internal control to ensure inter-assay repeatability a liver sample was included after every 4^{th} muscle sample in the experiment. PCR products were size separated by agarose gel electrophoresis (see Figure Appendix A 3.1) and band intensities determined using a phosphorimager and AIDA package (Appendix A 3.4.1(ii)c).

6.2c Statistical analysis

Insulin sensitivity was calculated from the glucose infusion rate (g / min) between 60 and 120 min of the euglycaemic clamp, dividing this infusion rate by body weight (kg) and mean insulin level (mU / l). As the distributions of plasma glucose, insulin and triglycerides measurements were skewed, $\log_e$ transformed variables were used in all analyses. All associations with GR were adjusted for 18S. To discount the effect of antihypertensive therapy, associations with 24 h mean ambulatory blood pressure were examined by censored normal regression, with the 6 men on antihypertensive therapy and the 3 with untreated values above 150 mmHg allocated to the top tertile (Statacorp 1997, Stata Statistical Software Release 5, College

Station, TX: Stata Corporation).. Statistical methods were advised by Professor P McKeigue, London School of Hygiene & Tropical Medicine.

6.3 Results

Results are shown in Table 6.1 and Figure 5.1. Increased skeletal muscle GR mRNA levels were significantly associated with higher insulin levels after a glucose load and raised blood pressure but not with plasma lipids, glucose or obesity. Associations with insulin sensitivity and fasting insulin levels were in the same direction but did not reach statistical significance in this small sample. There were also no associations between muscle GR mRNA levels and birthweight. In multiple regression analyses relationships with insulin sensitivity and blood pressure were independent of obesity.

Table 6.1 Associations of GR mRNA levels with cardiovascular risk factors

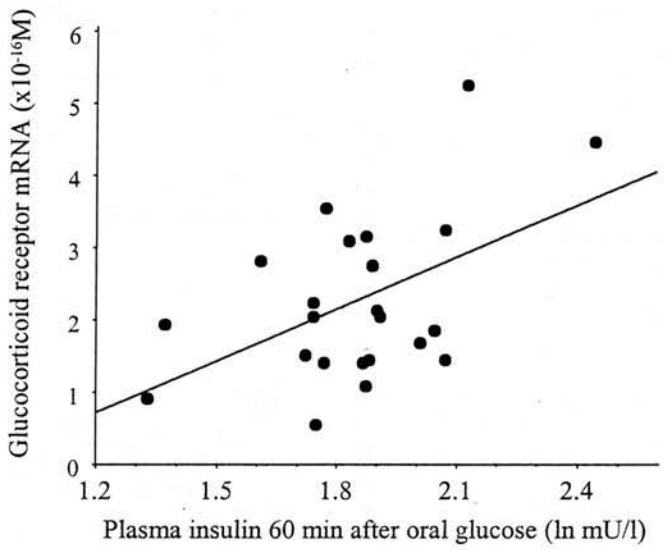
	Mean (sd)	*partial correlation coefficient*	*p-value*	*p-value adjusted for BMI*
[a]Fasting plasma glucose (mM)	5.5 (0.9)	-0.05	0.80	0.58
[a]Fasting plasma insulin (pM)	10.6 (8.2)	0.41	0.06	0.09
[a]Insulin 1h post glucose (pM)	74.2 (46.9)	0.53	0.01	0.02
[b]M/I ($mg.min^{-1}.kg^{-1}.(mU/l)^{-1}$)	5.2 (2.0)	-0.36	0.10	0.05
[c]24 h systolic blood pressure (mmHg)	142		0.05	0.05
[a]Triglycerides (mM)	1.2 (0.9)	0.26	0.24	0.30
BMI ($kg.m^{-2}$)	26.3 (3.3)	0.16	0.46	-
WHR	0.95 (0.05)	0.14	0.53	0.91
Birthweight (g)	3585 (423)	0.12	0.57	0.82

[a] variables log_e-transformed for analysis

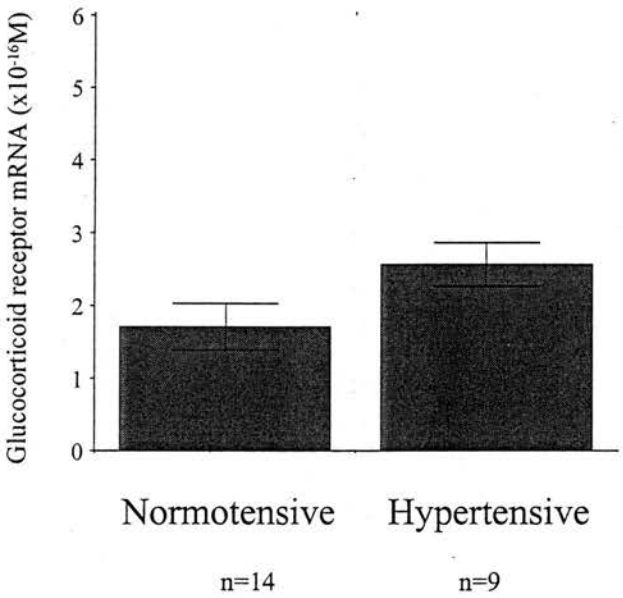
[b]glucose infusion rate ($g.min^{-1}$) between 60 and 120 min of the euglycaemic clamp divided by body weight (kg) and mean insulin level ($mU.l^{-1}$)

[c] median presented; using censored normal regression no partial correlation coefficient can be calculated

Figure 6.1
Associations of skeletal muscle GR mRNA levels with plasma insulin levels and blood pressure



partial correlation coefficient = 0.53
p=0.01 adjusted for 18S, p=0.02 adjusted for BMI



p=0.05 adjusted for 18S, p=0.05 adjusted for BMI

6.4 Discussion

These data suggest that men with insulin resistance and hypertension have increased GR mRNA levels and, by inference, increased numbers of GR in skeletal muscle. This is consistent with a recent report, as yet published only as an abstract, in skeletal muscle myoblasts cultured from biopsies obtained from men with insulin resistance (Whorwood *et al.* 1998). GR mediate diverse effects on insulin sensitivity (in liver, adipose tissue, and skeletal muscle) and blood pressure (in kidney, blood vessels, and brain). Increased receptor number in these sites could contribute to the association between features of the Metabolic Syndrome, and thus may explain previous observations of enhanced *in vivo* responsiveness to glucocorticoids (Walker *et al.* 1998b). This could be an important mechanism underlying the pathophysiology of insulin resistance and larger studies will be needed to determine whether the observations extend to subjects of low birthweight.

It may seem surprising that despite the previously observed modestly elevated circulating glucocorticoids in subjects with the Metabolic Syndrome (Phillips *et al.* 1998; Phillips *et al.* 2000), and the findings of Chapter 3, peripheral GR expression was elevated in men with hypertension and dyslipidaemia, since GR levels are often negatively auto-regulated by circulating glucocorticoids. Indeed, if receptor expression were similarly increased in central negative feedback sites, lower circulating cortisol levels to compensate for peripheral hypersensitivity would be expected. However, it appears likely that the dysregulation of GR expression is tissue-specific. For example, cortisol levels are higher, not lower, in subjects with the Metabolic Syndrome (Phillips *et al.* 1998; Phillips *et al.* 2000), and there were no differences in central feedback sensitivity demonstrated in men with the Metabolic Syndrome in Chapter 3. In addition, not all aspects of Cushing's syndrome are recapitulated in men with increased expression of GR in muscle; these men are not obese and do not have fasting hyperglycaemia, suggesting perhaps that the adipose tissue and liver are spared from this effect. Finally, the findings of increased peripheral GR but no change in central GR somewhat parallel the animal model of insulin resistance induced by prenatal exposure of rats to dexamethasone, where

dysregulation of GR expression is indeed tissue-specific, with increased GR mRNA in liver but decreased GR mRNA in central negative feedback sites (Nyirenda *et al.* 1998).

There are several potential mechanisms for dysregulation of GR expression. Polymorphisms of the GR gene have been associated with *in vivo* responsiveness to glucocorticoids (Panarelli *et al.* 1998) and with insulin resistance (Weaver *et al.* 1992) and may be in allelic association with variations in the tissue-specific promoter regions (Walker *et al.* 1998). One mechanism allowing differential regulation of GR expression is effects upon one or more multiple alternate promoters of the GR gene as occurs in rat and mouse (McCormick *et al.* 2000). Moreover, tissue-specific exon 1 usage is altered by perinatal environmental manipulations (McCormick *et al.* 2000). Whether the human GR gene is also regulated by more than one promoter is explored in Chapter 7.

Chapter 7

Multiple alternate first exons of the human GR gene: a mechanism for tissue-specific regulation of GR expression

7.1 Introduction

Thus far the results suggest that in men with the Metabolic Syndrome sensitivity to glucocorticoids is not the same in all tissues. Glucocorticoid sensitivity is increased in peripheral tissues (e.g. in skeletal muscle, Chapter 6) due to increased GR expression, but not in central tissues responsible for negative feedback (there was no difference in plasma cortisol following dexamethasone suppression, Chapter 3). Likewise in the offspring of rats treated with dexamethasone *in utero*, there is a similar pattern of tissue-specific GR expression, namely increased peripheral (hepatic) GR expression (Nyirenda *et al.* 1998) but decreased central (hippocampal) GR expression (Levitt *et al.* 1996). One mechanism for tissue-specificity, which allows differential GR regulation and has been reported for other members of the steroid receptor family, is the use of multiple and tissue-specific promoters (Kastner *et al.* 1990; Kwak *et al.* 1993; Flouriot *et al.* 1998; Zennaro *et al.* 1996).

The GR gene spans at least 110-kb and contains 8 coding exons (exons 2-9) and a non-coding exon 1 (Hollenberg *et al.* 1985; Encio and Detera-Wadleigh 1991; Zong *et al.* 1990). The human (Encio and Detera-Wadleigh 1991; Zong *et al.* 1990), mouse (Strähle *et al.* 1992; Cole 1992; Chen *et al.* 1999a; Chen *et al.* 1999b), and rat (Gearing *et al.* 1993; McCormick *et al.* 2000) GR gene promoter regions have been cloned and partially characterised. Rat GR mRNA exhibits considerable heterogeneity at the 5' ends (Gearing *et al.* 1993; McCormick *et al.* 2000). At least 11 alternate exon 1 sequences (1_1-1_{11}), are present in the rat GR gene (Gearing *et al.* 1993; McCormick *et al.* 2000); 5 of these correspond to those identified in the mouse GR gene (Strähle *et al.* 1992, Cole 1992) and one corresponds to that published for the human GR gene (Zong *et al.* 1990). Each alternate exon 1 is spliced from a fixed 3' donor site to the same 5' acceptor site in exon 2. The amino acid sequence of GR itself is unaffected by splicing of alternate exons 1 as there is an in-frame stop codon immediately upstream of the translation initiation site in exon 2. This complex

organisation of the 5'-end of the GR gene may reflect the need for diverse tissue-specific regulation and allows differential regulation of GR in specific cell types.

The use of alternative promoters in gene regulation provides an efficient and flexible means of controlling complex patterns of gene expression and thus is a potential mechanism for programming of gene expression. In rat tissues, two of the alternate exons (exons 1_6 and 1_{10}) are expressed in all tissues examined, and are together present in 77-87% of total GR mRNA (McCormick *et al.* 2000). The remaining GR mRNA transcripts contain tissue-specific alternate first exons. Tissue-specific first exon usage is altered by perinatal environmental manipulations. Postnatal handling of pups before weaning, which permanently increases hippocampal GR and attenuates stress responses (Francis *et al.* 1996; Liu *et al.* 1997), selectively elevates GR mRNA containing the hippocampus-specific exon 1_7 (McCormick *et al.* 2000). In prenatal glucocorticoid exposure, although total hepatic GR expression is increased (Nyirenda *et al.* 1998), the proportion of hepatic GR mRNA containing the predominant exon 1_{10} falls, suggesting that one or more of the minor exon 1 containing species is programmed to increase by the manipulation in early life.

Most of the alternate exons 1 in the rat GR gene lie within a 3-kb CpG island, upstream of exon 2, that exhibits substantial promoter activity in transfected cells. As this region is highly conserved between rat and human (Zong *et al.* 1990; Govindan *et al.* 1991), it is likely that the human GR gene also uses alternate promoters. Indeed recent evidence suggests the existence of at least two other alternate exons 1 within the human GR gene (Breslin and Vedeckis 1998; V Lyons & K Chapman, personal communication). Therefore the aim was to investigate whether human GR mRNA also exhibits 5'-heterogeneity.

7.2 Methods

7.2a 5'-RACE (Rapid Amplification of cDNA Ends)-PCR

5'-RACE is a procedure for amplification of nucleic sequences from a messenger RNA template between a defined internal site and unknown sequences at the 5'-end of the mRNA (Frohman 1993).

5'-RACE-PCR was performed using a commercial kit (Life Technologies Ltd.) according to the manufacturer's instructions with minor modifications (Appendix A 3.4.2) on total RNA isolated from human liver and peripheral blood mononuclear cells (Appendix A 2.7).

First strand cDNA synthesis of total RNA was carried out using a primer complementary to exon 2 of the human GR gene (GSP1) (Appendix A 3.4.2a). The first strand product was purified from unincorporated dNTPs and GSP1 (Appendix A 3.4.2b). TdT (terminal deoxynucleotidyl transferase) was used to add a homopolymeric dC tail to the 3' ends of the cDNA (Appendix A 3.4.2c). Tailed cDNA was then amplified by 35 cycles of PCR amplification (Appendix A 3.4.2d) using a nested gene-specific primer (GSP2) which anneals 3' to GSP1 (Appendix A 2.6a) and an 'anchor' primer (allowing amplification from the homopolymeric tail, supplied with the kit). To increase specificity a nested PCR was carried out on the products of the first PCR reaction, under the same conditions but using a 'universal anchor primer' (from kit) and GSP3 primer, which anneals 3' to GSP2 (Appendix A 2.6a). PCR products from nested PCR reactions were cloned into pGEM-T easy (Appendix A 3.3c), and sequenced using GSP3 (Appendix A 3.4.5).

7.2b RLM-RACE (RNA Ligase Mediated Rapid Amplification of cDNA Ends)

The major limitation of the above procedure is that there is no selection for amplification of fragments corresponding to the actual 5' ends of mRNA: all cDNAs are acceptable templates in the reaction. Additionally the PCR step favours amplification of less than full-length products, and thus usually produces a heterogeneous population of amplified products. RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) is an improvement to the classic RACE technique as it is designed to amplify cDNA only from full-length capped mRNA (Shaefer 1995).

Because of this, 5'-RACE-PCR was also performed on the human liver RNA using a First Choice RLM-RACE kit (Ambion Inc.) according to manufacturer's instructions (Appendix A 3.4.3).

RNA was firstly treated with calf intestinal phosphatase (CIP) to remove 5' phosphates from molecules such as ribosomal RNA, fragmented mRNA, tRNA and contaminating genomic DNA (Appendix A 3.4.3(i)a). The RNA was then treated with Tobacco Acid Pyrophosphatase (TAP) to remove the cap structure from full-length mRNA, leaving a 5' monophosphate (Appendix A 3.4.3(i)b). A synthetic RNA adapter, that can ligate only to decapped (full-length) RNA molecules with a 5' phosphate end, was ligated to CIP/TAP-treated RNA (Appendix A 3.4.3(i)c).

Reverse Transcription (RT) of ligated RNA was carried out (Appendix A 3.4.3(ii). Two rounds of 35 cycles of PCR amplification were performed (Appendix A 3.4.3(iii)). In the first 'outer' PCR, 1μl RT product was used in a reaction containing outer RNA adapter primer (from kit), and outer gene specific primer (GSP2 Appendix A 2.6a). A second 'inner' PCR was carried out on the products of the first PCR reaction, under the same conditions but with primers inner RNA adapter primer (from kit) and inner gene specific primer (GSP3 Appendix A 2.6a). PCR products from 'inner' nested PCR reactions were cloned and sequenced as above (and Appendices A 3.3c and A 3.4.5).

7.2c Reverse Transcription-PCR (RT-PCR)

To identify other novel human exon 1 species RT-PCR was performed on total RNA extracted from human liver, brain, fat and muscle (Appendix A 2.7) using primers corresponding to sequences predicted to be present based on sequence comparison with the rat GR gene (Appendix A 2.6d and see Figures 7.1a and 7.1b).

RT was performed using a commercial kit (Promega U.K. Ltd.) (Appendix A 3.4.4a). 35 cycles of PCR amplification of the RT product were performed (Appendix A 3.4.4b) using a 5'-primer (i.e. primers corresponding to rat exons 1_4, 1_5, 1_6, 1_7, 1_{10}, Appendix A 2.6a) and a 3'-primer complementary to exon 2 (GSP3 Appendix A 2.6a). An aliquot of the reaction mixture was analysed by gel electrophoresis (Appendix A 3.1a).

```
        1     AGGATTAGGTGGAGCTGCGGCAGCCTCCCGCCCGTGTCAGGAGCTGGCA
       50     AGCGATGTCTACCTGTGTGTGCGCAAAAGTTACCTCCCCAAACCCTAAA
1₄    100     CCCACACAGCACAACCTTTCCCAGA.TCACAAAAATCATAATCTGTGTC
      150     TGCACAAGGTAG.GAGGCTCGGTCCCGGCATCGTCGAAGCCTTCCCGAC
      200     GCGGCGAGCTGGGGAAGGGAGCTGGGGCGGGGGCTTCCCGCACGGGCAC
      250     CCCTCGCCCCACGCCCTCTCCTTTCTCAGGACGGACCACGAGTTCCCTT
      300     CCCCTTGGACTGAGGGGGAAGCTCCTAACAGGAACATCTGTAGGGAGTT
      350     GAACGCTGGCATTTTAAAGCTGCCTGTATTTTGTTTTATTTGTAGGGGC
      400     AGGGGTCCTATGAACGTGATAGGGTGAGCAACGCACAGAGTCGAGGGCA
      450     GCAAATGTCAAGATTCGGGGGTGGGGCCTGCACCGGGAACTTGGACGCG
      500     GGCCCTGGCCGGGGTGGAAGAAGAGGTCAGGAGTTCGG.AAGGGGGCTA
      550     TATTTCGCCAGCAACTTACTATTTCGCCTGCAACTTGCTTTTAAGCCTG
      600     CCGCCCCCTGCTTTCCTTAATCATAATAATAAAAAAAAAGTGCAAAGAA
1₅    650     ATCCAGCTCGCTGGAGGTTTTGCATTTGGCGTGCAACTTCCTTCGAGTG
      700     TGAGCACATTGGGCGGGAGGGGTGGGGGTTGAACTTGGCAGGCGGCGCC
      750     TCCTTCTGCCGCCGCCGCCGCCTCGCAGACTCGGGGAAGAGGGTGGGGG
      800     ACGGTCGGGGCGCGGGGGAGGGTGGGTTCTGCTTTGCAACTTCTCTC.C
      850     CAGTGCGAGAGCGCGGCGGCGGCAGCTGAAGACCCGGCCGCCCAGATGA
1₆    900     TGCGGTGGTGGGGGGACCTGCCGGCACGC.CGACTCCCCCCGGGCCCAAA
      950     GTACGTATGCGCCGACCCCCGCTATCCCGTCCCTTCCCTGAAGCCTCCC
     1000     CAGAGGGCGTGTCAGGCCGCCCGGCCCCGAGCGCGGCCGAGACGCTGCG
     1050     GCACCGTTTCCGTGCAACCCCGTAGCCCCTTTCGAAGTGACACACTTCA
     1100     CGCAACTCGGCCCGGCGGCGGCGGCGCGGGCCACTCACGCAGCTCAGCC
     1150     GCGGGAGGCGCCCCGGCTCTTGTGGCCCGCCCGCTGTCACCGCAGGGGC
     1200     ACTCGGCGCTTGCCGCCAAGGGGCAGAGCGAGCTCCCGAGTGGGTCTGG
     1250     AGCCGCGGAGCTGGGCGGGGGCGGGAAGGAGGTAGCGAGAAAAGAAACT
1₇   1300     GGAGAAACTCGGTGGCCCTCTTAACGCCGCCCCAGAGAGACCAGGTCGG
     1350     CCCCCGCCCTGCCGCCGCCACCCTTTTTCCTGGGGAGTTGGGGGCGGGG
     1400     GGCGAAGCGCGGCGCACCGGGCGGGGCGGCCACGCCAGGGGACGCGGGC
     1450     GTGCAGGCG.CGTCGGGGCCGGGGTGGCGGGGCCCGCGCGGAGGGCG.T
     1500     GGGGGCAGGGACCGCGGGCGCCCCTGCAGTTGCCAAGCGTCACCAACAG
     1550     GTTGCATCGTTCCCCGCGCCGCCGCGCGGCCCCTCGGGCGGGGAGCGGC
     1600     CGGGGGTGGAGTGGGAGCGCGTGTGTGCGAGTGTGTGC.CGCCGTGGCG
     1650     CCGCCTCCACGCCGCTCCCCGCTCGGTCCCGCTCGCTCGCCCAGGCCGG
     1700     GCTGCCCTTTCGTGTCCGCGCTCTCTTCCCTC.CGCCGCCGCCTCCTCC
     1750     ATTTTGCGAGCTCGTGTCTGTGACGGGAGCCCGAGTCACCGCCTGCCGT
     1800     CGGGGACGGATTCTGTGGGTGGAAGGAGACCCGCACCGGAGCGGCCGAA
     1850     GCAGCTGGGACCGGGACGGGCACGCGCGCCCGGAAGCCCCGACCCGCGG
     1900     AGCCCGGCGCGGGGCGGAGGGCTGGCTTGTCAGCTGGGCAATGGGAGAC
     1950     TTTCTTAAATAGGGCTCTCCCCCCACCCATGGAGAAAGGGGCGGCTGTT
     2000     TACTTCCTTTTTTTAGAAAAAAAAAATATATTTCCCTCCTGCTCCTTCT
     2050     GCGTTCACAAGCTAAGTTGTTTATCTCGGCTGCGGCGGGAACTGCGGAC
     2100     GGTGGCGGGCGAGCGGCTCCTCTGCCAGAGGTAAGAAGCGAGGCGGGAG
     2150     GGGGCCGGGGCGCGCTCGCTCCCCCGAGGTGCCGCTGGGACCGGAGACA
     2155     ACTCGGGGGCCGCCGCGGGAGCCTACAAACTTTTATTAGCCTCGGGGAG
     2200     TGGGGGTGGGGGGCTGGCAAGGGCCGGGCGACGGTGACGAAAGGGTAGC
     2255     GCGCGGGTGACAGCGCTGGCCTCTTCCTCTCCCTCCGCCGGCGTCCCTG
     2300     GCCGGGCCGAGGGGGAGGAACCTGACCTCGGACGGCGAGCGGAGCCCTG
     2350     TCGAACTCCGGGGCTTCGAGCCTCTCATTCTCGCGGGAATCCTGGCCTC
     2400     TTTTCTCCCCCTAGTGTCCCCTTTCCTCCAAGGGGGTCGCCCGACACCC
     2450     GTTTTCGTGGTGAGACTAAGCCGCGTCTGAATTTTACTCGCCCGAATAT
     2500     TTCACCCACCCCGCCCGAGCGCGAGCCC
```

Figure 7.1a Sequence of 5'-DNA flanking exon 2 of the human GR gene Showing position of exons 1

Nucleotides are numbered as in published sequence
(Accession no. M32284, Zong *et al.* 1990)
Nucleotides in ***Bold italic*** font represent position of primers
Nucleotides which are underlined indicate corresponding sequence in rat GR gene where exons 1 identified (as by McCormick *et al.* 2000)

```
          1     CCGGGCCCAAAGTACGTATGCGCCGACCCCCGCTATCCCGTCCCTTCCC
         50     TGAAGCCTCCCCAGAGGCGTGTCAGGCCGCCCGCCCGAGCGCGGCGAGA
        100     CGCTGCGCACCGTTTCCGTGCAACCCCGTAGCCCCTTTCGAAGTGACAC
        150     ACTTCACGCAACTCGGCCCGGCGGCGGGCGGCGCGCGACTCACGCAGCT
        200     CAGCCGCGGGAGGGCCCCGGCTCTTGTGGCCCGCCCGCTGTCACCCGCA
        250     GGCACTGGCGGCGCTTGCAGAGGGGCAGAGCGAGCTCCCGAGTGGGTCT
        300     GGAGCCGCGGACGTGGGCGGGGCGGGAAGGAGGTAGCGAGAAAAGAAAC
        350     TGGAGAAACTCGGTGGCCCTCTTAACGCCGCCCCAGAGAGACCAGGTCG
        400     GCCCCCGCCGCTGCCGCCGCCACCCCTTTTCCTGGGGAGTTGGGGGCGG
        450     GGGGCGAACGCGGCGCACCGGGCGGGGCGGCCACCGAGGGGACGCGGCG
        500     TGCAGGCGCAGTCGGGGCCGGGGTGGCGGGGCCCGCGCGGAGGGCGTGG
1_8     550     GGGCAGGGACCGCGGGCGCCCCTGCAGTTGCCAAGCGTCACCAACAGGT
        600     TGCATCGTTCCCCGCCGGCCGGCGCGGCCCTCGGGCGGGGAGCGGCCGG
        650     GGGTGGAGTGGGAGCGCGTGTGTGCGAGTGTGTGCGCCGTGCGCCGCCT
        700     CCACCGCTCCCGCTCGGTCCCGCTCGCTCCGCCAGGCCGGGCTGCCCTT
        750     TGCGTGTCCGCGCTCTCTTCCCTCCGCCGCCGCCTCCTCCATTTTGCGA
1_9     800     GCTCGTGTCTGTGACGGGAGCCCGAGTCACCGCACTGCCGTCGGGGACG
        850     GATTCTGTGGGTGGAAGGAGACGCCGCAGCCGGAGCGGCCGAAGCAGCT
        900     GGGACCGGGACGGGGCACGCGCGCCCGGAAGCCCCGACCCGCGGAGCCC
        950     GGCGCGGGGCGGAGGGCTGGCTTGTCAGCTGGGCAATGGGAGACTTTCT
1_10   1000     TAAATAGGGCTCTCCCCCCCACCCATGGAGAAAGGGGCGGCTGTTTACTT
       1050     CCTTTTTTTAGAAAAAAAAAATATATTTCCCTCCTGCTCCTTCTGCGTT
       1100     CACAAGCTAAGTTGTTTATCTGGCTGCGGCGGGAACTGCGGACGGTGGC
       1150     GGGCGAGCGGCTCCTCTGCCAGAGGTAAGAAGCGAGGCGGGAGGGGGCC
       1200     GGGGCGCGCTCGCTCCCCCGAGGTGCCGCTGGGACCGGAGACAACTCGG
       1250     GGGCCGCCGCGGGAGCCTACAAACTTTTATTAGCCTCGGGGACTGGGGG
       1300     TGGGGGGCTGGCAAGGGCCGGGCGACGGTGACGAAAGGGCAGCGGGCGG
       1350     GTGACAGCGCTGGCCTCTTCCTCTCCCTCCGCCGGCGTCCCTGGCCGGG
       1400     CCGAGGGGGAGGAACCTGACCTCGGACGGCGAGCGAGCCCTGTCGAACT
       1450     GCCGGGGCTTCGAGCCTCTCATTCTCGCGGGAATCCTGGCCTCTTTTCT
       1500     CCCCCTAGTGTCCCCTTTCCTCCAAGGGGGTCGCCCGACACCCGTTTTC
       1550     GTGGTGAACGCT.AAGCCGCGTCTGAATTTTACTCGCCCGAATATTGTG
1_11   1600     CACGCCACCCCGCGGCGCCCGAGCGCGAGCCCGGG
```

Figure 7.1b Sequence of 5'-DNA flanking exon 2 of the human GR gene showing positions of exons 1

Nucleotides are numbered as in published sequence
(Accession no. S68374 Govindan *et al.* 1991)
Nucleotides in ***Bold italic*** font represent position of primers
Nucleotides which are underlined indicate corresponding sequence in rat GR gene where exons 1 identified (as by McCormick *et al.* 2000)

7.3 Results

7.3.1 5'-RACE-PCR

In the majority of products identified by 5'-RACE-PCR, the 5'-end was located in exon 2. Some products contained exon 1 sequence in addition to that of exon 2. Of these, two independent 5'-RACE products were obtained from liver and peripheral blood mononuclear cells RNA, and one from brain, deriving from independent cDNAs generated in the initial reverse transcription reactions. The 5'-ends of the cDNAs terminated at different positions in exon 1, indicating that they are unlikely to be generated by PCR from the same initial tailed cDNA. Clones from liver, peripheral blood mononuclear cells and brain contained the exon 1 sequence previously identified in human GR mRNA (now termed human 1C, Breslin and Vedeckis 1998) and which corresponds to exon 1_{10} of the rat and exon 1C of the mouse GR gene. In addition, clones from liver and peripheral blood mononuclear cells contained exon 1 sequence corresponding to exon 1_6 of the rat and exon 1B of the mouse GR gene, and hence termed human exon 1B (Breslin and Vedeckis 1998).

7.3.2 RLM-RACE

Sequence analysis of individual clones revealed the presence of exons 1 corresponding to published human exon 1C (equivalent to rat exon 1_{10}) in liver. The exon 1C clones differed in length suggesting alternate transcription start sites. The most 5'-sequence obtained corresponds to nucleotide position –950 according to the published human GR cDNA sequence (see Figure 7.1b), and is further 5' than the published 1C sequence (Govindan *et al.* 1991).

7.3.3 RT-PCR

Exons 1 corresponding to rat exons 1_4, 1_5, and 1_7, and human exons 1B and 1C (equivalent to rat exons 1_6 and 1_{10}) were identified in RNA extracted from human liver, skeletal muscle, fat and brain. Figure 7.2 shows a representative agarose gel of novel exons 1 identified in skeletal muscle. Figure 7.3 summarises these results showing a schematic representation of the human GR gene promoter region as compared with the published rat and mouse GR gene promoter regions.

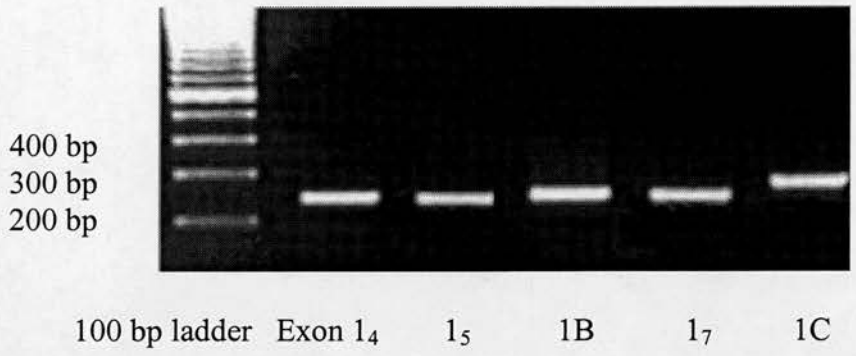


Figure 7.2 Representative agarose gel showing novel human exons 1 identified by RT-PCR in total RNA isolated from human skeletal muscle

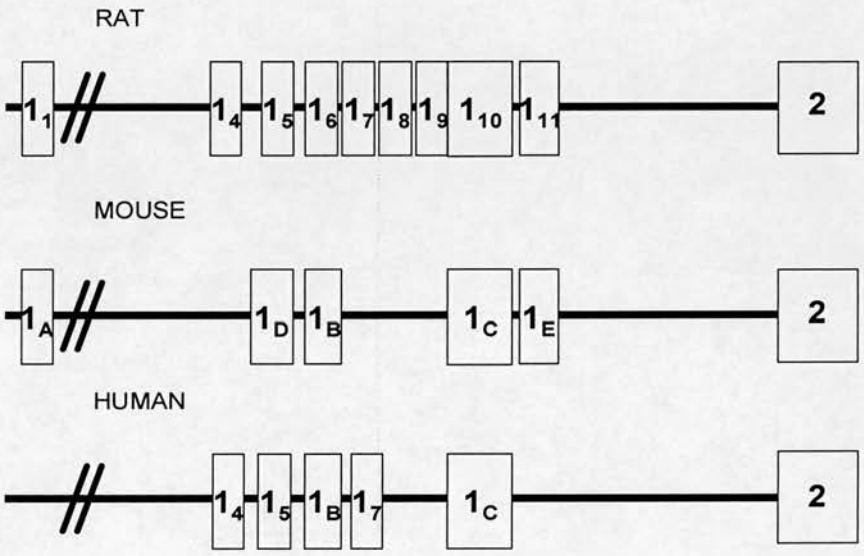


Figure 7.3 Schematic representation (not to scale) of the human GR gene promoter.

Figure shows published human exons 1B and 1C (Encio and Detera-Wadleigh 1991, Zong *et al.* 1990*)* and novel exons 1 identified compared with the published rat (McCormick *et al.* 2000) and mouse (Strähle *et al.* 1992, Cole 1992) GR gene promoter regions.

The Bcl I restriction fragment-length polymorphism (Panarelli *et al.* 1998) associated with abdominal obesity, insulin resistance and elevated blood pressure, lies within intron 1 i.e. upstream of exon 2

7.4 Discussion

The findings show that there is more than one exon 1 in the human GR gene. Sequence analysis of individual clones generated from the 5'-RACE-PCR experiments, revealed the presence of exon 1 sequences previously identified in human GR mRNA as exon 1B (Breslin and Vedeckis 1998) and exon 1 (Encio and Detera-Wadleigh 1991; Zong *et al.* 1990), now designated exon 1C (Breslin and Vedeckis 1998) (equivalent to rat exons 1_6 and 1_{10}) in liver and peripheral blood mononuclear cells. Exon 1C was also identified in the brain tissue. Previous work from the laboratory has identified exons 1B and 1C in GR mRNA from human peripheral blood mononuclear cells (V Lyons & K Chapman, personal communication). Human exon 1B is homologous to the rat exon 1_6 (Gearing *et al.* 1993; McCormick *et al.* 2000) and mouse exon 1B (Strähle *et al.* 1992; Cole 1992) and is present in the published human GR gene sequence at position –873 to –925 (Zong *et al.* 1990). Human exon 1C is homologous to rat exon 1_{10} (Gearing *et al.* 1993; McCormick *et al.* 2000) and mouse exon 1C (Strähle *et al.* 1992; Cole 1992) and is present in the published human GR gene sequence at position –1111 to –1035 (Govindan *et al.* 1991). Other novel exons 1 were identified by RT-PCR using primers corresponding to sequences predicted to be present based on sequence comparison with the rat GR gene. Thus exons 1 corresponding to rat exons 1_4, 1_5, and 1_7, and human exons 1B and 1C (equivalent to rat exons 1_6 and 1_{10}) were identified in all tissues examined (see Figure 7.2).

GR is expressed in virtually all cell types, but levels of GR vary between tissues, within tissues and during development. This is important as the level of GR expression is key in determining glucocorticoid action. The wide variation in GR expression both spatially and temporally suggests complex regulation of the GR gene. Use of alternative promoters in gene regulation provides an efficient means of controlling complex patterns of gene expression. Thus alternate promoter usage may be a key mechanism underlying tissue-specific responses to hormone, and is also a potential mechanism for programming of gene expression. In addition, production of mRNAs with different untranslated 5'-leader sequences by alternative promoter

usage can affect gene expression by variations in stability or translation efficiency of these mRNAs (Kozak 1991).

The standard 5'-RACE-PCR method has limitations. As there is no selection for amplification of fragments corresponding to the actual 5' ends of mRNA, all cDNAs are acceptable templates in the reaction. Additionally, the PCR step selects the most efficient (smallest) amplicons, favouring amplification of less than full-length products. Indeed most of the transcripts generated by this method were short, only extending into exon 2. The alternative method of RLM-RACE, is designed to amplify cDNA only from full-length capped mRNA. Any first strand cDNA molecules that do not extend all the way to the 5' end of the adapter will not yield a product in the PCR (since these targets lack the adapter-specific primer binding sites). Use of this technique generated exon 1C clones of differing length. Although shorter transcripts could represent truncated artifacts of reverse transcription and amplification, it is most likely that they represent alternate transcription start sites. CpG islands are frequently associated with multiple transcription initiation sites (Koller *et al.* 1991; Ye *et al.* 1993) and at least four transcription start sites have been mapped for the published human exon 1 (Zong *et al.* 1990; Encio and Detera-Wadleigh 1991). The regions flanking exon 1 may be important in determining differential promoter activity as they contain multiple GC boxes, the recognition site of the SP1 transcription factor (Zong *et al.* 1990; Encio and Detera-Wadleigh 1991).

The region of the CpG island in which 8 of the 11 alternate exons 1 of the rat GR gene lie shows moderate conservation with the corresponding human GR gene sequence (~70% identity over the CpG island; nucleotides –1600 to –4220) (Zong *et al.* 1990; Govindan *et al.* 1991). It seems likely that more alternative exons 1 could exist within the CpG island.

In rat tissues, the abundance of alternate promoters varies in different tissues allowing tissue-specific regulation of GR gene expression. Although the alternate promoters are ubiquitously expressed in human tissues, further work will establish whether the abundance of the different mRNA species varies in different tissues.

Chapter 8

Conclusion

The role of the HPA axis in programming of adult disease

The idea that stimuli or insults during critical or sensitive periods in early life have lifelong consequences is well established in developmental biology and has been termed 'programming'. Recently the phenomenon of programming has been used to explain the epidemiological data showing that small size at birth or in infancy is associated with an increased risk of adverse health outcomes in adult life including diabetes, hypertension and ischaemic heart disease. Such observations have led to the 'fetal origins hypothesis' whereby environmental factors acting *in utero* or in the early postnatal period permanently alter the development of organs and lead to later organ dysfunction and disease (Barker 1998). Despite early criticisms, the associations between parameters of early growth and adult disease have been reproduced throughout the world, and research is now centred on identifying the underlying factors that are involved in programming of disease.

Recent studies suggest the HPA axis plays an important role. In animal studies adverse influences in prenatal or early post-natal life permanently alter the biological and behavioural responses in the adult offspring through long-term changes in central and peripheral sensitivity to glucocorticoids (Benediktsson *et al.* 1993; Langley and Jackson 1994; Levitt *et al.* 1996; Nyirenda *et al.* 1998). In humans, glucocorticoid excess (in Cushing's syndrome) is associated with the Metabolic Syndrome and subsequent cardiovascular disease and therefore it is plausible that more subtle variations in glucocorticoid activity contribute to the pathogenesis of risk factors for cardiovascular disease in the population. Indeed, higher fasting morning plasma cortisol concentrations are associated with higher blood pressure, plasma glucose and triglyceride concentrations, and also with lower birthweight (Phillips *et al.* 1998; Phillips *et al.* 2000). These preliminary studies led to the hypothesis that events in early life permanently alter or 'programme' cortisol secretion and that this may be a fundamental mechanism underlying the association between low birthweight and the development of metabolic disorders and cardiovascular disease in adult life.

The data in Chapter 3 show that an underlying mechanism mediating the association between elevated fasting plasma cortisol and low birthweight and / or the Metabolic Syndrome is activation of the HPA axis with increased cortisol response to exogenous $ACTH_{1-24}$ and increased excretion of urinary cortisol metabolites. This activation of the HPA axis has now been observed in a population of younger individuals of known birthweight and early clinical features of the Metabolic Syndrome (Levitt *et al.* 2000).

Animals prenatally exposed to dexamethasone have elevated basal and stress-induced plasma glucocorticoid levels (Benediktsson *et al.* 1993; Langley-Evans *et al.* 1994; Levitt *et al.* 1996; Nyirenda *et al.* 1998) and subtle changes in HPA axis regulation. GR and MR expression are decreased in the hippocampus (Levitt *et al.* 1996), one of the most sensitive targets for glucocorticoid negative feedback effects in rodents, but increased in the amygdala (Welberg *et al.* 2000), a site of positive modulation of HPA axis activity. In contrast in humans there was no evidence of altered central feedback sensitivity to low dose dexamethasone in men with low birthweight or the Metabolic Syndrome (Chapter 3). However, at low doses dexamethasone has limited access to the brain and so may only test the pituitary component of the negative feedback loop. Further studies are needed to explore the use of alternative glucocorticoids such as prednisolone or hydrocortisone in feedback regulation. Moreover, while the role of hippocampal MR in HPA axis regulation has been well established in rodents (de Kloet *et al.* 1998), little is known about its relevance in humans, although MR is known to be located in the human hippocampus (Seckl *et al.* 1991). Evidence supporting a physiological role for MR in human central feedback includes the observations that administration of MR antagonists (potassium canrenoate or spironolactone) increase plasma cortisol concentrations (Dodt *et al.* 1993; Young *et al.* 1998; Heuser *et al.* 2000; Arvat *et al.* 2001) whereas fludrocortisone suppresses plasma cortisol in healthy adults (Mangos *et al.* 2000). It remains possible that glucocorticoid feedback occurs via a centrally mediated MR mechanism and responses to MR antagonists such as spironolactone to increase HPA drive should be tested.

Alternatively, rather than impaired central negative feedback, the activation of the HPA axis could be due to increased forward drive to ACTH and cortisol secretion from higher centres. The lack of habituation to the stress of repeated venepuncture observed in diabetic subjects in Chapter 4 is consistent with this explanation. Interestingly recent rodent studies suggest habituation may be MR mediated (Cole *et al.* 2000). Such alterations in the central regulation of the HPA axis may be key in the development of glucose intolerance. Lack of habituation to stress and increased activation of the HPA axis in subjects with glucose intolerance also supports the hypothesis that chronic stress in man leads to development of cardiovascular risk factors. Similar variations in HPA axis activity may contribute to the observed relationships between psychosocial stress and subsequent cardiovascular disease (Brunner 1997). Methods which have been employed to test feed forward of the HPA axis (e.g. Trier Psychosocial Stress Test (Kirschbaum *et al.* 1993), combined dexamethasone-CRH test (Heuser *et al.* 1994)) may be useful in furthering understanding of activation of the HPA axis.

An alternative mechanism whereby alterations in glucocorticoid activity may contribute to the pathogenesis of risk factors for cardiovascular disease is through altered tissue responsiveness to glucocorticoids. In Chapter 6 increased expression of GR mRNA in skeletal muscle was associated with insulin resistance suggesting that this may be a key underlying mechanism. Further studies are needed to see whether increased peripheral sensitivity to glucocorticoids is also apparent in subjects of low birthweight.

Together the animal studies and results of Chapters 3 and 6 suggest that GR expression is tissue-specific. The finding of alternate promoters of the human GR gene in Chapter 7 provides a mechanism for tissue specific regulation of GR expression. Further work is needed to see whether levels of the different promoters vary in different tissues. This could provide a novel mechanism for tissue-specific manipulations of target organ response and drug design. Certainly therapeutic manipulation of the HPA axis itself is not an attractive target to improve insulin sensitivity. Unacceptable side effects similar to Addison's disease would be

anticipated if plasma cortisol was lowered due to the ubiquitous expression of steroid receptors. However, specificity of effects for drugs acting at other steroid receptors has been achieved. An example is raloxifene, a 'selective oestrogen receptor modulator' (SERM), which acts as an oestrogen antagonist in breast and endometrium but as an agonist in bone (MacGregor and Jordan 1998). Further understanding the tissue-specificity of cortisol action will be important in drug design to minimize the risk of adverse effects.

Data in this thesis suggest that in humans programmed changes in the regulation of the HPA axis with activation of the HPA axis and increased tissue sensitivity to glucocorticoids may have long term adverse effects. This is particularly important as glucocorticoids have extensive therapeutic use prenatally. For example they are used to accelerate lung maturation in pre-term labour (Crowley *et al.* 1990). In addition, glucocorticoids are used for prenatal therapy for fetuses at risk of congenital adrenal hyperplasia (Speiser and New 1994). Low birthweight has been reported in these babies (Pang *et al.* 1992), and problems with social and emotional behaviour in children treated with dexamethasone *in utero* have been observed (Trautman *et al.* 1995). It remains to be seen whether such manipulations also carry a risk of development of metabolic and cardiovascular diseases in adult life.

Appendix 1

Questionnaire

Questions 1-9 used in the study of dynamic testing of the HPA axis in Hertfordshire (Chapter 3); Question 9 used in the validation of the overnight low dose dexamethasone suppression test (Chapter 2)

I would like to ask you a few questions about any illnesses you may have suffered since we saw you last.

1.a	Have you ever been told by a doctor that you have diabetes?	Yes/No
1.b	At what age was it diagnosed?	.....
1.c	What treatment do you have for it? a. insulin injections b. tablets c. diet only	.....
2.a	Have you ever been diagnosed by a doctor as having a heart attack?	Yes/No
2.b	Have you ever been diagnosed by a doctor as having angina?	Yes/No
2.c	Have you ever had an operation to clear the arteries in your heart (coronary artery bypass graft or angioplasty)?	Yes/No
2.d	If yes, in what year?	.....
3.	Have you ever been diagnosed by a doctor as having a stroke?	Yes/No
4.	have you ever been treated by a doctor for high blood pressure?	Yes/No
5.	Have you had any other illness since we interviewed you last?	Yes/No
	If so, please tell us.....	

6. What regular medicines/pills/tablets/skin creams are you using?

In particular:

- have you taken any oral steroids, used steroid inhalers or used steroid containing creams or eye drops in the last year? Yes/No
- if yes, please give details below
- have you ever taken HRT? Yes/No
- if yes, what preparation? .....

and when did you take it? .....

PLEASE USE BLOCK CAPITALS

COPY NAMES DIRECTLY OFF BOTTLES IF POSSIBLE

1. _____
2. _____
3. _____
4. _____
5. _____
6. _____
7. _____
8. _____
9. _____
10. _____

7. I'd like to ask you whether you have drunk different types of alcoholic drinks in the last 12 months. I do not need to know about non-alcoholic drinks.

Do you ever drink alcohol? 0. No Go to Q8 _____
1. Yes **ask 7a) etc**

a) How often do you currently drink
Shandy or Low Alcohol Beer/Lager/Cider FFQ 1-7_____ > x1 _____

When you drink these how many pints would you normally have? _____
(if range give code mid-point; 1 average can =0.8 pints)

b) How often do you currently drink
Beer/Stout/Lager/Cider FFQ 1-7_____ > x1 _____

When you drink these how many pints would you normally have? _____
(if range give code mid-point)

c) How often do you currently drink
Low alcohol wine FFQ 1-7_____ > x1 _____

When you drink these how many glasses would you normally have? _____
(if range give code mid-point)

d) How often do you currently drink
Wine/Sherry/Martini/Cinzano FFQ 1-7_____ > x1 _____

When you drink these how many glasses would you normally have? _____
(if range give code mid-point)

e) How often do you currently drink
Spirits/Liqueurs FFQ 1-7_____ > x1 _____

When you drink these how many measures would you normally have? _____
(if range give code mid-point)

8.a Have you ever smoked regularly? (ie at least once a day for a year or more)

1. Yes – ask question 8.b
0. No

8.b How old were you wen you first smoked regularly? .....

8.c What is the most that you have ever smoked regularly? .....

1. Cigarettes _____ / day _____
2. Roll-ups _____ / day _____
3. Tobacco _____ / day _____
4. Cigars _____ / day _____

8.d Do you still smoke regularly?

1. Yes
2. No

8.e How old were you when you last smoked regularly? .....

9. Some of the tests we are doing at the clinic are affected by how you are feeling so I just need to ask you some questions about this:

Are you basically satisfied with your life?	YES/NO
Have you dropped many of your activities and interests?	YES/NO
Do you feel that your life is empty?	YES/NO
Do you often get bored?	YES/NO
Are you in good spirits most of the time?	YES/NO
Are you afraid that something bad is going to happen to you?	YES/NO
Do you feel happy most of the time?	YES/NO
Do you often feel helpless?	YES/NO
Do you prefer to stay at home, rather than going out and doing new things?	YES/NO
Do you feel you have more problems with memory than most?	YES/NO
Do you think it is wonderful to be alive now?	YES/NO
Do you feel pretty worthless the way you are now?	YES/NO
Do you feel full of energy?	YES/NO
Do you feel that your situation is hopeless?	YES/NO
Do you think that most people are better off than you are?	YES/NO
Have you needed to see your doctor because of depression or nerves in the last 2 years?	YES/NO

Appendix 2

Laboratory Materials

A 2.1 *Chemicals*

Agarose and low melting point agarose	Life Technologies Ltd., 3 Fountain Drive, Paisley PA3 9RF
Agar	Life Technologies Ltd.
Amfix fixative	H.A. West Ltd., 41 Watson Crescent, Edinburgh, EH11 1ES
Bactotryptone	Beckton Dickinson, Between Towns Road, Cowley, Oxford, OX4 3LY
Caesium chloride	Sigma Chemicals Ltd., Fancy Road, Poole, Dorset BH17 7NH
D19 developer	H.A. West Ltd.
DNA size markers (1kb ladder)	Life Technologies Ltd.
Ethanol	Hayman Ltd., 70 Eastways Industrial Park, Witham, Essex CM8 3YE
Nucleotide triphosphates	Amersham Pharmacia Biotech UK Ltd., Amersham Place, Little Chalfont, Bucks HP7 9NA
[α-^{33}P] ddNTPs	Amersham Pharmacia Biotech UK Ltd.
Phenol:chloroform:isoamyl alcohol 25:24: 1	Sigma Chemicals Ltd.
TRIzol reagent	Life Technologies Ltd.
Yeast extract	Beckton Dickinson

A 2.2 *Buffers and solutions*

Unless stated solutions were prepared with distilled water. All solutions, except those marked * were sterilized in an autoclave before use.

Alkaline SDS solution*	0.2 M NaOH, 1% SDS. Freshly prepared before use.
Deionised formamide*	15 g Amberlite ion exchange resin (BDH. Lutterworth, UK) mixed with 150 ml formamide for 1 h, then filtered twice to remove Amberlite
DEPC water	ultrapure water (500 ml) mixed with diethylpyrocarbonate (DEPC; 5 drops) and left for 1-24 h before autoclaving
DNA loading buffer*	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 25% Ficoll made up in DEPC-treated water
0.5 M EDTA (pH 8.0)	800 ml water added to 186.1 g $Na_2EDTA.2H_20$. pH adjusted to 8.0 with NaOH, volume adjusted to 1 litre
Formamide loading buffer*	0.34% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol, 20 mM EDTA in deionised formamide
GTE	50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0
10x MOPS buffer	0.2M 3-[N-morpholino]propanesulfonic acid, 50 mM Na acetate, 5 mM EDTA, pH 7.0
5 M potassium acetate	245.6 g potassium acetate dissolved in 300 ml water. Volume adjusted to 500 ml by adding 57.5 ml glacial acetic acid and 142.5 ml water

10x TBE buffer	108.9 g Tris, 55.7 g boric acid, 20 ml 0.5 M EDTA, volume adjusted to 1 litre with water
TE buffer	10 mM Tris-HCl (pH 8.0), 1 mM EDTA
1M Tris-HCl, pH 8.0	121.1 g Tris base in 800 ml water. pH adjusted to 8.0 with concentrated HCl and volume adjusted to 1 litre

A 2.3 *Bacterial media*

Luria-Bertoni (LB) broth	10 g bactotryptone, 5 g yeast extract, 5 g NaCl made up to 1 litre with distilled water and autoclaved immediately
LB-agar	3 g agar added to 300 ml LB broth before autoclaving. For plates, LB-agar melted in a microwave oven, cooled until warm, 100 μg/ml ampicillin added, and poured into 100 mm petri dishes.

A 2.4 *Plasmids*

pGEMT-easy	Promega U.K. Ltd., Delta House, Chilworth Research Centre, Southampton SO16 7NS
E. coli JM109 cells	Promega U.K. Ltd.

A 2.5 *Enzymes and buffers*

*SP*6, *T*7, *Sal*I, *Nco*I, *Eco*RI and 10x restriction buffers	Promega U.K. Ltd.
High activity *T*4 DNA ligase and 10x buffer	Roche Diagnostic Ltd., Bell Lane, Lewis, East Sussex BN7 1LG
Taq polymerase and 10x buffer	Roche Diagnostic Ltd.
*Taq*Bead™ hot start polymerase wax beads and Thermophilic DNA polymerase 10x reaction buffer, Mg-free	Promega UK Ltd.

A 2.6 *Oligonucleotides*

A 2.6a *5'-RACE-PCR* — Oswel DNA Service, University of Southampton, Southampton SO 16 7PX

GSP1	5'-AAGGGATGCTGTATTCA-3'
GSP2	5'-ACTCCAAATCCTTCAAGAGGTCA-3'
GSP3	5'-TTGGAATCTGCCTGAGAAGC-3'

A 2.6b *Primers used for construction of 'competitor' template and used in competitive quantitative RT-PCR assay*

Operon Technologies, Inc., 1000 Atlantic Avenue, Alameda, CA 94501

(i) GR primers

5' primer hGR-1	5'-GAC ATT TTG CAG GAT TTG GAG-3'
3' primer hGR-2	5'-GCT TAC ATC TGG TCT CAT GCT-3'
3' linker hGR-3	5'-TGG TCT CAT GCT CTT GGC ACC TAT TCC AAT T-3'

(ii) 18S primers

5' primer h18S-1	5'-CGG AAC TGA GGC CAT GAT TA-3'
3' primer h18S-2	5'-GGA CAT CTA AGG GCA TCA CA-3'
3' linker h18S-3	5'-GGG CAT CAC ACG TAA CTA GT-3'

A 2.6c *Sequencing*

SP6	5'-TGT AAT ACG ACT CAC TAT AG-3'
T7	5'-TGT AAT ACG ACT CAC TAT AG-3'

A 2.6d *RT-PCR* (See also Figure 7.1a and 7.1b) — VH Bio Ltd. P.O Box 7, Gosforth, Newcastle upon Tyne, NE 3 4DB

Exon 1_4	5'-TAA ACC CAC ACA GCA CAA CC-3'
Exon 1_5	5'-TCG CTG GAG GTT TTG CAT TTG-3'
Exon 1B	5'-CAG ATG ATG CGG TGG T-3'
Exon 1_7	5'-GAA ACT GGA GAA ACT CGG TGG-3'
Exon 1C	5'-TGC GTT CAC AAG CTA AGT TG-3'

A 2.7 *RNA*

Total RNA isolated from human liver (supplied by V. Lyons, isolated from tissue freshly obtained by P. Hadoke from the Liver Transplant Programme, Edinburgh).

Total RNA isolated from human peripheral blood mononuclear cells (supplied by V. Lyons, prepared from buffycoat from Blood Transfusion Service, Edinburgh).

Total RNA isolated from human brain (supplied by D. Brown, prepared from post-mortem specimen).

Subcutaneous adipose tissue supplied by P. Hadoke. RNA prepared by TRIzol method (A 3.2).

Skeletal muscle (from Uppsala study – Chapter 6. RNA prepared by TRIzol method (A 3.2).)

A.2.8 *Others*

Autoradiographic film	H.A.West Ltd.
First-strand cDNA synthesis kit	Amersham Pharmacia Biotech UK Ltd.
First choice RLM-RACE Kit	Ambion, Inc.
Genius Thermal Cycler	Thistle Scientific Ltd., Unit 48, Greenhill Business Centre Coltswood Road, Coatbridge ML5 2AA
GlassmaxDNA isolation system	Life Technologies Ltd.
Hybaid Recovery DNA Purification Kit II	Hybaid Ltd., Action Court, Ashford Road, Ashford, Middlesex TW15 1XB
Microcon centrifugal filter device	Millipore Corp., Bedford MA 01730 USA
5'-RACE system version 2.0	Life Technologies Ltd.
Reverse Transcription System	Promega U.K. Ltd.
RiboProbe In Vitro Transcription Systems	Promega U.K. Ltd.
Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit	Amersham Pharmacia Biotech UK Ltd.

Appendix 3

Laboratory methods

A 3.1 *Gel electrophoresis*

a) *Agarose gels*

Agarose gels were prepared by adding solid agarose (for analysis of PCR products) or low melting point agarose (to gel purify DNA fragments) to 0.5x TBE 0.8-2.5% (w/v), boiling in a microwave oven, and adding 0.5 μg/ml ethidium bromide. The gel was poured into a gel mould with an appropriate comb and once set, submerged in 0.5x TBE buffer in an electrophoresis tank; Horizon 58 (50 ml gel) or Horizon 11.54 (100 ml gel) (Life Technologies Ltd.). 1kb DNA markers and samples containing 1 μl DNA loading buffer were loaded into the wells and electrophoresed at 140V. DNA was visualised by UV transillumination at $\lambda = 254$ nm, imaged using an Appligene Imager and recorded on Seikosha video printer paper.

b) *RNA gels*

Integrity of total RNA was confirmed by denaturing agarose gel electrophoresis. Before use, gel trays, tanks and combs were scrubbed in hot soapy water, soaked in 0.1 M NaOH for 30 min and rinsed with ultrapure water. A 1% (w/v) gel was prepared by melting 0.25 g agarose in 18 ml DEPC water in a microwave oven for 30 s. Once cooled slightly, 40% (w/v) formaldehyde (2 ml) and 10x MOPS buffer (10 ml) were added and the mixture poured into a gel mould with appropriately sized combs in place. Once set, the gel was aged by submerging in an electrophoresis tank in 1x MOPS buffer for 15 min. RNA (20 μg) was prepared for electrophoresis by adding 40% (w/v) formaldehyde (2.5 μl), 10x MOPS buffer (2.5 μl) and deionised formamide (10 μl) in a total volume of 25 μl. The sample was mixed and RNA denatured by incubating at 65° C for 15 min. Ethidium bromide was added to the loading buffer (1 μl per 50 μl loading buffer) and 2 μl of this mix added to each sample of denatured RNA. The RNA was loaded onto the wells on the gel and electrophoresed for 20-30 min at 100V. RNA was visualised by UV transillumination ($\lambda = 254$ nm) and photographed as before.

c) *Sequencing gels*

Prior to use, two glass plates (45 cm x 35 cm) were scrubbed with detergent and rinsed with distilled water and then absolute ethanol. 0.3 mm spacers were inserted between the two clean plates, the edges clamped with bulldog clips and sealed with 2% agarose (w/v). 42 g urea was dissolved in 15 ml 40% acylamide:bisacrylamide (19:1) and 4 ml 20x glycerol tolerant buffer. The solution was made up to 100 ml with distilled water and filtered through Whatman #1 filter paper. 600 µl 10% ammonium persulphate (w/v) was added and mixed. 40 µl TEMED was added and the gel mix cast immediately and left overnight to polymerise. The gel was assembled into the electrophoresis tank (Kodak Biomax STS 45I) using 0.8x glycerol tolerant buffer as running buffer. The gel was pre-run at 1800 V for 30 min and the wells flushed with buffer before loading the samples, which had been denatured in formamide loading buffer at 95°C for 3 min. Gels were typically run at 1800 V and then transferred to Whatman 3MM paper and vacuum-dried at -80°C (BioRad 583 gel drier). The gel was exposed to autoradiographic film overnight and then developed.

A 3.2 *Ribonucleic acid (RNA) preparation*

Total RNA was extracted using the TRIzol method. Tissue was disrupted by grinding in a pestle and mortar under liquid nitrogen. 1 ml TRIzol reagent was added to up to 100 mg ground tissue. Samples were further disrupted using syringes with needles of decreasing gauge (19-, 23- and 25- gauge) and then incubated for 5 min at room temperature. 0.2 ml chloroform was added, samples incubated at room temperature for 3 min and centrifuged in a microcentrifuge at 14,000 rpm for 15 min at 4°C. The aqueous phase was transferred to a fresh eppendorf tube and the RNA precipitated by addition of 0.5 ml isopropanol, incubation at room temperature for 10 min and centrifugation at 4°C for 10 min at 14,000 rpm. The supernatant was removed with a drawn-out glass pasteur pipette and pellets washed with 1 ml cold (4°C) 75% ethanol. After vortexing, samples were centrifuged at 4°C for 5 min at 7,500 rpm. The supernatant was removed and pellets air-dried for 5 min at room temperature. Pellets were re-suspended in 20 µl DEPC water by incubating at 65°C for 15 min and vortexing vigorously.

A 3.3 *Manipulation of DNA*

a) *Restriction digests*

Plasmid DNA was digested with the appropriate restriction enzyme (10 U) in 1x restriction enzyme buffer in a total volume of 10-40 µl for 1-2 h at 37°C (or 40°C depending on enzyme). Digestion of the DNA was confirmed by agarose gel electrophoresis, comparing against uncut plasmid.

b) *DNA fragment recovery*

DNA fragments were purified using a commercially available kit (Hybaid Recovery DNA Purification Kit II). Fragments were firstly resolved on a 1-2 % low melting point agarose gel run at 90V, visualised under UV light (λ = 365 nm) (to prevent damage to DNA) and then excised from the gel using a scalpel. Gel slices were placed in a spin filter, 400 µl of resuspension binding buffer added, the sample placed at 55°C for 5 min, and then centrifuged for 30 s at 14,000 rpm. 500 µl Wash Solution was added to the spin filter and centrifuged as before. This wash step was repeated, the pellet dried by a further 1 min centrifugation and the spin filter transferred to a new catch tube. 20 µl Elution Solution was added, the binding matrix/DNA re-suspended by flicking and DNA recovered by centrifugation as before. Recovery of DNA was assessed by agarose gel electrophoresis of a 1-2 µl aliquot of DNA solution.

c) *Cloning of DNA*

(i) *DNA ligation*

Fragments recovered from low melting point agarose gels were ligated using 1-5 U *T4* DNA ligase in 2x supplied buffer in a final volume of 10 µl (typically using 2-4 µl (approximately 50 ng) of insert DNA, and 0.5-1 µl (approximately 100 ng) vector DNA). Reactions were incubated at room temperature for 1 h before transforming into commercially prepared competent JM109 *E. coli*.

(ii) *Transformations*

JM109 cells were removed from storage at -80°C and allowed to thaw on ice. 50 µl cells was transferred to a pre-chilled 1.5 ml glass test tube, 200-500 ng DNA added

and the mixture gently flicked to mix. The reaction was placed on ice for 10 min, heat-shocked at 42°C for 50 s and allowed to recover on ice for 2 min. 200 µl of LB was added, the cells placed in a rotating incubator at 37°C for 30 min and plated on LB agar (containing 40 µl 50 mg/ml X-GAL, 40 µl 0.1M IPTG and 100 µg/ml ampicillin). The plates were incubated upside down at 37°C overnight.

(iii) *Screening of clones*

(iii)a *Minipreps*

A single transformed bacterial colony was selected and incubated overnight in a rotating incubator at 37°C in 2 ml LB containing 100 µg/ml ampicillin. Cultures were centrifuged at 14,000 rpm for 1 min in a microcentrifuge and the pellets re-suspended in 100 µl cold GTE buffer. 200 µl fresh alkaline SDS was added, vortexed and placed on ice for 10 min. 150 µl cold 5M potassium acetate (pH 4.8) was added, vortexed and placed on ice for a further 10 min. Following centrifugation at 14,000 rpm for 5 min, the supernatant was transferred to fresh eppendorf tubes. 225 µl chloroform and 225 µl Tris-HCl saturated phenol were added, the mixture vortexed and centrifuged for 2 min. The supernatant was transferred to a fresh eppendorf tube, 2 volumes of absolute ethanol added, vortexed and incubated at room temperature for 5 min. After centrifugation at 14,000 rpm for 5 min, the supernatant was removed with a drawn-out glass pasteur pipette and the pellet left to air dry for 10 min. The pellet was re-suspended in 50 µl TE containing 50 ng RNase A and stored at -20°C.

(iii)b *Large scale plasmid DNA preparation*

Single colonies of transformants containing the required plasmid were added to 500 ml LB containing 100 µg/ml ampicillin and incubated overnight at 37°C in a shaking incubator. Cultures were centrifuged at 6,000 rpm for 5 min at 4°C in a Beckman J14 rotor, and the supernatant discarded. The cell pellet was re-suspended in 12 ml cold GTE buffer, mixed with 24 ml fresh alkaline SDS, and placed on ice for at least 10 min. 16 ml of cold 5 M potassium acetate was added, mixed gently, and placed on ice for a further 10 min prior to centrifugation at 6,000 rpm for 10 min at 4°C in a

Beckman J14 rotor. The supernatant was filtered through sterile gauze to remove the precipitate. 32 ml isopropanol was added to the filtrate and the mixture left at room temperature for 30 min to precipitate the DNA. The DNA was pelleted by centrifugation at 10,000 rpm for 3 min at 4°C in a Beckman J14 rotor, and the supernatant discarded. The DNA pellet was air-dried and re-suspended in 2.2 ml TE buffer. 3 g CsCl was added, dissolved and 100 µl of ethidium bromide (10 mg/ml) added. The DNA solution was transferred to a 3 ml Beckman Quickseal ultracentrifuge tubes, tubes topped up with CsCl/TE solution and centrifuged for either 4 h at 100,000 rpm, or for 16 to 20 h at 70,000 rpm in a TLA100.3 rotor in a Beckman Optima TLX Ultracentrifuge. The plasmid DNA band was removed using a needle and syringe through the tube wall and transferred to a fresh tube. CsCl/TE was added to fill the tube and the tube was centrifuged as before. The plasmid DNA band was again removed and the ethidium bromide removed by extracting repeatedly with isopropanol until the pink colour disappeared. Plasmid DNA was then dialysed against three changes of 2 l TE at 4°C. The concentration and quality (ratio of absorbances at λ = 260 and 280 nm) of plasmid DNA were determined using a GeneQuant spectrophotometer (Pharmacia Biotech). Plasmid DNA was stored at -20°C.

A 3.4 *Polymerase chain reaction (PCR)- based methods*

A 3.4.1 *Competitive quantitative RT-PCR assay*

(i) *Construction of synthetic RNA competitors*

RNA competitors for human GR mRNA and human 18S mRNA (used as an internal control for variability in target RNA) were synthesised separately but under the same conditions (Benchmarks 1994). Both competitors contain 83 base pair (bp) deletions to distinguish the PCR products derived from endogenous and synthetic RNAs.

(i) a) *Reverse Transcription (RT)*

RT was performed using a kit (Amersham Pharmacia Biotech U.K. Ltd.). 200 ng total RNA isolated from human liver (Appendix A 2.7) was incubated in a total

volume of 8 µl at 65°C for 10 min, centrifuged briefly, and placed on ice. 7 µl of a reverse transcription mix containing 5 µl reverse transcription buffer (contains reverse transcriptase, RNAguard™, RNase/DNase-free BSA, dATP, dCTP, dGTP, and dTTP in aqueous buffer), 1 µl 200 mM dithiothreitol (DTT) and 1 µl (0.2 µg) random hexadeoxynucleotides were added (total volume of 15 µl), and the reaction incubated at 37°C for 1 h.

(i) b) *PCR*

7.5 µl RT product was used in a 50 µl reaction mixture containing 5 µl *Taq* buffer (contains 100 mM Tris-HCl, 15 mM $MgCl_2$, 500mM KCl), 1 µl dNTP (10mM of each nucleotide), 10 µM primer 1 (hGR-1 or h18S-1) (Appendix A 2.6b), 10 µM primer 2 (hGR-2 or h18S-2) (Appendix A 2.6b) and 0.5 µl *Taq* polymerase. The reaction mixture was overlaid with 2 drops of mineral oil, and incubated at 94°C for 3 min before 30 cycles of PCR amplification (94°C 40 s; 54°C 40 s; 72°C 40 s) were performed. The reaction was incubated at 72°C for 10 min following the last cycle of PCR. A 5 µl aliquot of the reaction mixture was analysed by gel electrophoresis (Appendix A 3.1a) to confirm the presence of one band of the expected size (635 bp for GR, 613 bp for 18S).

To create the internal deletion 1 µl of the first PCR product was used as a template for the first re-amplification step. PCR was carried out under the same conditions but the 3' primer (primer 1) was replaced by the internal linker primer (hGR-3 or h18S-3) (Appendix A 2.6b). The 5' end of the internal linker primer is identical to the 3' end of the 3' primer (match of 12 bases for GR and 10 bases for 18S) and thus serves as a 'linker' between the 3' and 5' primers in the second re-amplification step. A 5 µl aliquot of the reaction mixture was analysed by gel electrophoresis to confirm the presence of one band of the expected size (531 bp for GR and 511 bp for 18S).

In the second re-amplification, 1µl of the first re-amplification step PCR product (containing the 531 bp GR or 511 bp 18S fragment) was re-amplified under the same PCR conditions using the original 3' primer (primer 1). In both re-amplification

steps the same 5' primer (primer 2) was used. A 5 µl aliquot of the PCR product was analysed by gel electrophoresis to confirm the presence of only one band (543 bp for GR, 521 bp for 18S). This PCR fragment thus contains an internal deletion (83 bp for both GR and 18S), with identical 5' and 3' ends to the original fragment and the same DNA sequence except for the missing 83 bp.

(i) c) *Isolation of PCR fragment and plasmid preparation*

The PCR product was run on a 2% low melting point gel, the fragment cut out and the DNA purified (Appendix A 3.3b), ligated into pGEMT easy vector (Appendix A 3.3c(i)) and transformed into JM109 cells (Appendix A 3.3c(ii)). Small-scale preparation of plasmid DNA was performed (Appendix A 3.3c(iii)a) and sequenced using *SP6* and *T7* to check the orientation of the insert (Appendix A 3.4.5). Large-scale plasmid DNA preparations were made so that there was sufficient competitor for the whole experiment (Appendix A 3.3c(iii)b).

(i) d) *Linearization of template*

Linear GR/18S template was prepared by digesting 10 µg of the DNA from above in a 50 µl reaction with 30 U restriction enzyme for 1 h (*Sal*I at 37°C for GR, *Nco*I 40°C for 18S). Linearization was confirmed by agarose gel electrophoresis.

(i) e) *In vitro transcription*

In vitro transcription was carried out using a commercial kit (Promega U.K. Ltd.). 250 ng linearised template was incubated for 1 h at 37°C in a 50 µl reaction containing 10 µl 5x transcription buffer (contains 200 mM Tris-HCl (pH 7.9), 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 5 µl 100 mM DTT, 50 U RNasin, 10 µl rNTPs (ACGU 2.5mM), 30 U polymerase (*T7* or *SP6* according to orientation of insert). 2 U DNase was added and incubated at 37°C for a further 15 min to digest the DNA.

The synthesised competitor RNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform/isoamyl alcohol (24:1) and then passed through a microcon centrifuge filter YM100 to remove free nucleotides. RNA was

precipitated overnight at -80°C by adding 0.1x volume 3 M sodium acetate (pH 5.5) and 2.5x volume absolute ethanol. This was spun at 12,000 rpm for 15 min, the pellet washed with 75% ethanol, dried and re-suspended in 10 µl water.

RNA was quantitated on a GeneQuant spectrophotometer (Pharmacia Biotech) and by agarose gel electrophoresis against an RNA mass ladder. The RNA competitors were stored at -80°C.

(i) f) *Calculation of competitor concentration*

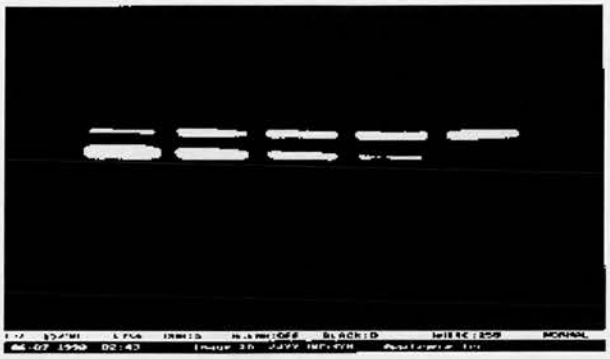
The concentration of the competitor in moles (M) was calculated by dividing the concentration of RNA (µg/µl) quantitated from GeneQuant by the molecular weight of the competitor (where molecular weight = average molecular weight of nucleotide (330) x number of nucleotides in fragment (calculated from final PCR product size + number of base pairs from vector).

A 3.4.1 (ii) *Competitive quantitative RT-PCR assay*

The range of competitor concentrations for use in the assay was determined using 100 ng total RNA from liver (for assay methods see below), firstly by diluting competitors over a 10-fold range, and then narrowing to a 4-fold range (see Figure A 3.1). The inter-assay coefficient of variation was 12%. As an additional internal control to ensure inter-assay repeatability a liver sample was included after every 4th muscle sample in the experiment.

(ii) a) *RT*

RT was carried out as above (Appendix A 3.4.1(i)a). For each sample, 4 separate RT reactions were set up over a 4-fold range of serial dilutions of the two competitors (for GR (2 x 10^{-11}M, 1 x 10^{-11}M, 0.5 x 10^{-11}M, 0.25 x 10^{-11}M); for 18S (2 x 10^{-7}M, 1 x 10^{-7}M, 0.5 x 10^{-7}M, 0.25 x 10^{-7}M)). Thus each reaction contained 100 ng target RNA + 1 µl of each competitor (Tube 1, GR 2 x 10^{-11}M, 18S 2 x 10^{-7}M; Tube 2 GR 1 x 10^{-11}M, 18S 1 x 10^{-7}M etc.).



Target RNA (ng)	100	100	100	100	100
GR Competitor (M) 1 x	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}

Figure A 3.1 Example of agarose gel from competitive-quantitative RT-PCR assay on human liver

Gel shows increasing size of bands for the 100 ng target RNA used in the assay against the decreasing band size of the serial 10-fold dilutions of GR competitor. Band intensities are determined using a phosphorimager and AIDA package and the ratio of target band to competitor band, corrected for size differences calculated. The log ratio of band intensities is plotted against the log of competitor concentration and the amount of target mRNA obtained at the equivalence point of 1.

(ii) b) *PCR*

PCRs for GR and 18S were performed separately using the same conditions as above (Appendix A 3.4.1(i)b). For GR 7.5 µl RT product was used with 10 µM primer 1 (hGR-1) and 10 µM primer 2 (hGR-2), and for 18S 5 µl RT product was used with 10 µM primer 1 (h18S-1) and 10 µM primer 2 (h18S-2).

(ii) c) *Quantification of data*

PCR products were size separated by agarose gel electrophoresis. The gel was exposed to a Fuji phosphor screen and analysed using a FujiFilm FLA-2000 phosphorimager. Background was set by placing a linebox at the level of interest and measuring the intensity (mm). Band areas were enclosed within a rectangle and the intensity within the band determined by 3D densitometry (AIDA package),

subtracting background. The ratio of target band to competitor band, corrected for size differences, was calculated (using Sigma Plot 4.0). The log ratio of band intensities was plotted against the log of competitor concentration and the amount of target mRNA obtained at the equivalence point of 1.

A 3.4.2 *5'-RACE (Rapid Amplification of cDNA Ends) - PCR*

a) *First strand cDNA synthesis*

5'-RACE-PCR was performed using a commercial kit (Life Technologies Ltd.) according to the manufacturer's instructions with minor modifications. 2.5 pmol GSP1 (Appendix A 2.6a), a primer complementary to human exon 2, was annealed to 5 µg total RNA in a total volume of 15 µl by denaturing at 70°C for 10 min and then placing on ice for 1 min. First strand cDNA synthesis of GR cDNA was carried out in a 25 µl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 400 µM dNTPs (all supplied with kit), incubating at 42°C for 1 min, adding 200 U SuperScript II reverse transcriptase and incubating for a further 50 min. Reverse transcriptase was inactivated by incubation at 70°C for 15 min, and adding 1 µl RNase mix before further incubation at 37°C for 30 min.

b) *cDNA purification*

cDNA was purified using a GlassMAX DNA Isolation Spin Cartridge. 100 µl of ultrapure water was equilibrated to 65°C and the binding solution (6M NaI) equilibrated to room temperature. 120 µl binding solution was added to the cDNA and the mixture transferred to a GlassMAX spin cartridge. The spin cartridge was centrifuged at 13,000 rpm for 20 s and the flow-through discarded. 400 µl cold (4°C) 1x wash buffer was added to the spin cartridge and centrifuged at 13,000 rpm for 20 s. This wash step was repeated three times. The cartridge was then washed twice with 400 µl cold (4°C) 70% ethanol and centrifuged at 13,000 rpm for 1 min after the final wash. The spin cartridge was transferred into a sample recovery tube and 50 µl of the pre-heated 65°C water added. cDNA was eluted by centrifugation at 13,000 rpm for 20 s. The purified cDNA was dried using a Speedvac (Savant) to a volume of 10 µl.

c) *Terminal deoxynucleotidyl transferase (TdT) tailing of cDNA*

10 µl purified cDNA was tailed with dC in a 25 µl reaction containing 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 1.5 mM $MgCl_2$ (supplied as 5x tailing buffer with kit), and 200 µM dCTP. The reaction mix was incubated for 2 min at 94°C, and chilled on ice for 1 min. 10 U TdT was added and the reaction incubated for 10 min at 37°C. TdT was inactivated by incubating at 65°C for 10 min.

d) *PCR*

Two rounds of PCR were performed to increase specificity. In the first round, 10 µl dC-tailed cDNA was used in a reaction with 400 nM 'anchor' primer (5'-CUACACUACUAGGCCACGCGGTCGACTAGTACGGG-IIGGGIIGGGIIG-3', from kit), 400 nM GSP2 primer (Appendix A 2.6a), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 µM dNTPs in a total volume of 50 µl. Reactions were overlaid with 2 drops of mineral oil and incubated at 94°C for 5 min before adding 2.5 U *Taq* DNA Polymerase. 35 cycles of PCR amplification (96°C, 45 s; 50°C, 40 s; 72°C, 1 min 30s) were performed, followed by incubation at 72°C for 10 min. A nested PCR was carried out on the products of the first PCR reaction, under the same conditions but with 400 nM each of primers 'universal anchor primer' (5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3', from kit) and GSP3 (Appendix A 2.6.1).

A 3.4.3 *RLM (RNA Ligase Mediated Rapid Amplification of cDNA Ends) -RACE*

5'-RACE-PCR was also performed using a First Choice RLM-RACE kit (Ambion Inc.) according to manufacturer's instructions.

(i) *RNA processing*

(i) a) *Calf intestinal phosphatase (CIP) treatment*

10 µg total RNA was incubated at 37°C for 1 h in a 20 µl reaction containing 2 µl 10x CIP buffer and 2 µl CIP (supplied with kit). The CIP reaction was terminated by adding 15 µl ammonium acetate solution and the RNA was extracted with phenol:chloroform.

(i) b) *Tobacco acid pyrophosphatase (TAP) treatment*

5 µl CIP-treated RNA was incubated at 37°C for 1 h in a 10 µl reaction containing 10x TAP buffer and 1 µl TAP (supplied with kit).

(i) c) *RNA adapter ligation*

A synthetic RNA adapter was ligated to 2 µl CIP/TAP-treated RNA by incubating at 37°C for 1 h in a 10 µl reaction containing 10x RNA ligase buffer, 1 µl RNA adapter and 2.5 U *T*4 RNA ligase (all supplied with kit).

(ii) *RT*

2 µl ligated RNA was reverse transcribed by incubating at 42°C for 1 h in a 20 µl reaction containing 4 µl dNTPs, 2 µl random decamers, 2 µl 10x RT-PCR buffer, 10 U RNase inhibitor and 1 µl MMLV reverse transcriptase.

(iii) *PCR*

Two rounds of nested PCR were performed. In the first 'outer' PCR, 1 µl RT product was used in a reaction containing 2 µl outer RNA adapter primer (from kit), 2 µl outer gene specific primer (GSP2, Appendix A 2.6a), 5 µl 10x RT-PCR buffer, and 4 µl dNTPs in a total volume of 50 µl. Reactions were incubated at 94°C for 3 min before adding 1.25 U *Taq* DNA Polymerase. 35 cycles of PCR amplification (94°C, 1 min; 60°C, 1 min; 72°C, 1 min) were performed, followed by incubation at 72°C for 7 min. A second 'inner' PCR was carried out on the products of the first PCR reaction, under the same conditions but with 2 µl each of primers inner RNA adapter primer (from kit) and inner gene specific primer (GSP3, Appendix A 2.6a).

A 3.4.4 *Reverse Transcription (RT) - PCR*

a) *RT*

RT was performed using a commercial kit (Promega U.K. Ltd.) on 5 µg total RNA in a total volume of 40 µl containing 4 µl reverse transcription buffer (contains 100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 1% Triton X®-100), 25 mM $MgCl_2$, 10 mM dNTP, 40 U recombinant RNasin ribonuclease inhibitor, and 1 µg oligo(dT)

primer, adding 40 U AMV reverse transcriptase and incubating at 42°C for 45 min. RT was inactivated by incubation at 99°C for 5 min and 4°C for 5 min.

b) *PCR*

5 µl RT product was used in a 50 µl reaction mixture containing 5 µl Taq buffer (contains 100 mM Tris-HCl, 500 mM KCl, Triton®X-100), 3 µl $MgCl_2$ (25 mM), 1 µl dNTP (10 mM of each nucleotide), 40 pM 5'-primer (i.e. primers corresponding to rat exons 1_4, 1_5, 1_6, 1_7, 1_{10}, Appendix A 2.6d) and 40 pM 3'-primer (GSP3 Appendix A 2.6a). One *Taq*Bead™ hot start polymerase wax bead was added, the reactions overlaid with 2 drops of mineral oil and incubated at 95°C for 4 min 10 s. 35 cycles of PCR amplification (95°C 50 s; 50°C 45 s; 72°C 1 min 30 s) were performed, followed by incubation at 72°C for 5 min.

A 3.4.5 *Sequencing*

DNA sequencing was performed using a Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech UK Ltd.). Termination mixes for each ddNTP were prepared by mixing 2 µl dGTP nucleotide master mix and 0.5 µl [α-^{33}P] ddNTP (G, A, T, or C; one of each per sequencing reaction). A reaction master mix was prepared containing 2 µl reaction buffer (contains 260 mM Tris-HCl, pH 9.5, 65 mM $MgCl_2$), 1 µl DNA, 0.05 pmol primer (Appendix A 2.6.3), 13 µl distilled water and 8 U Thermo Sequenase polymerase. 4.5 µl reaction mix was added to each termination mix. 35 cycles of 95°C, 30 s; 55°C, 30 s; 72°C 1 min 30 s were performed and upon completion of the cycling program, 4 µl 'stop' solution (contains 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added and the reactions stored at -20°C. Samples were resolved on 6% glycerol tolerant sequencing polyacrylamide gels (Appendix A 3.1c).

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Permission to publish

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Reproducibility of the low dose dexamethasone suppression test: comparison between direct plasma and salivary cortisol assays

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Summary

BACKGROUND The low dose dexamethasone suppression test (DST) has been used to detect subtle variations in the feedback suppression of the hypothalamic–pituitary–adrenal axis, which may contribute to the pathogenesis of several diseases including depression, the metabolic syndrome and coronary artery disease. Little is known about the reproducibility of this test, or whether the test can be combined with analysis of salivary cortisol which would offer a significant advantage over plasma in population studies.

SUBJECTS AND DESIGN A low dose DST was carried out in 29 healthy subjects (14 men, 15 women), aged 24–54 (mean 35·1) years, on two separate occasions 1–10 weeks apart. Following the administration of 0·25 mg dexamethasone (DXM) at 2200 h, plasma and saliva were sampled at 0830 h the next day. Cortisol was measured by radioimmunoassay in plasma and time-resolved immunofluorescent assay ('DELFIA') in saliva. Bland–Altman plots were produced for post-DXM plasma and salivary cortisol measures and used to derive a coefficient of repeatability for each measure, which describes the range of cortisol measurements within which 95% of repeated measurements will fall.

RESULTS The baseline, pre-DXM cortisol concentrations were far more variable for saliva (mean 16·5, range 4·4–34 nmol/l) than for plasma (mean 407·5; range 232–958 nmol/l). Following DXM both measurements showed an approximately 30% suppression from baseline but the variability of salivary cortisol was much greater. From the Bland–Altman plots the 95% range for the differences about their mean was calculated and used as an indication of repeatability. For plasma 95% of differences were within 0·78 log units, indicating that a repeated measurement was approximately half as small or twice as large as the first. For saliva 95% of differences were within 1·64, indicating that a repeated measurement was approximately five times as small or five times as large as the first.

CONCLUSIONS Assessment of dexamethasone suppression by salivary cortisol measurement is far less repeatable than the use of plasma cortisol. In the context of field studies of dexamethasone suppression, salivary cortisol measurements may only be appropriate for large numbers of subjects.

Although the adverse health effects of excess glucocorticoids in patients with Cushing's disease or during treatment with exogenous corticosteroids are well known, subtle increases in the circulating concentrations of these hormones have been implicated in the aetiology of several common disorders including central obesity, depression, the metabolic syndrome and possibly coronary artery disease (Carroll *et al.*, 1981; Bjorntorp, 1993; Ljung *et al.*, 1966; Brunner, 1997). These changes in glucocorticoid exposure may be mediated by alterations in the sensitivity of the central feedback control of the hypothalmic–pituitary–adrenal axis (HPAA). These are demonstrable by administration of the synthetic glucocorticoid, dexamethasone (DXM), at night, which results in the suppression of plasma cortisol concentrations the following morning.

The conventional protocol for the overnight dexamethasone suppression test (DST) (Liddle, 1960) depends on the administration of between 1 and 2 mg DXM and is designed to identify patients with Cushing's syndrome. However, the use of a much lower DXM dose, e.g. 0·25 mg (Best *et al.*, 1997) may be more informative in distinguishing physiological variations in HPAA activity. Recently it has been suggested that the very low dose test could be further refined by measuring

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the overnight suppression of salivary rather than plasma cortisol. Measurement of salivary cortisol levels would offer several potential advantages, enabling it to become a useful epidemiological tool for population studies of HPAA function (Landon *et al.*, 1982). These include simplicity of sample collection, allowing the test to be performed at home, the avoidance of venepuncture which results in stress-induced hypercortisolaemia and that salivary measurements reflect free plasma cortisol levels which are less influenced by changes in corticosteroid binding globulin. Because of the interest in studying the HPAA in large groups of subjects at risk of obesity or the metabolic syndrome, we have assessed the reproducibility of the very low dose DST by comparing cortisol suppression in saliva and plasma in 29 healthy subjects.

Methods

The study group consisted of 29 healthy subjects, 14 men and 15 women. None of them had a history of endocrine disease. They were taking no medication and none had received steroid treatment systemically or topically within the previous 6 months. The study was approved by the Southampton and South West Hampshire Health Authority and University of Southampton Joint Ethical Committee.

In each subject two low dose DXM tests were performed at least 1 week but no more than 10 weeks apart. On the first day the subjects attended after fasting for 12 h and saliva and blood samples were collected at 0830 h. Between 2 and 4 ml of saliva was collected into a salivette tube (Sarstedt Ltd, Leicester, UK). The subjects took 0·25 mg DXM at 2200 h the same night and attended the following morning after a further 12-h overnight fast for collection of saliva and blood samples at 0830 h. Plasma and saliva samples were separated by centrifugation and stored at −40°C until analysis. The subjects' height was measured with a portable stadiometer (CMS weighing equipment, Camden, London, UK) and weight with a SECA scale (SECA Ltd, Birmingham, UK). Body mass index was calculated as the weight (kg) divided by the height (m) squared. Waist and hip circumferences were measured with a steel tape measure, at the level of the umbilicus and the greater trochanters, respectively. The waist to hip ratio was calculated as a measure of central obesity. The subjects were also asked to complete the 10-item General Health Questionnaire (cut-off score for depression = score > 5), a previously validated questionnaire for detecting depression (Yesavage *et al.*, 1983). Cortisol was measured in plasma by radioimmunoassay (Guildhay antisera, Moore *et al.*, 1985). Cortisol and cortisone were measured in saliva by time-resolved immunofluorescent assay ('DELFIA', Wood *et al.*, 1997). The coefficients of variation (CV) were 6·3% at 300 nmol/l for plasma-based and 8·5% at 12 nmol/l for saliva-based assays.

Statistical analysis

As the distributions of cortisol measurements were highly skewed, $\log_e$-transformed values were used in all analyses. Bland–Altman plots (the differences between two repeated measurements for each subject against the mean of those measurements) were produced for post-DXM plasma and salivary cortisol measures (Bland & Altman, 1986). From these the 95% range for the differences about their mean was calculated and this used as an indication of repeatability. Fractional suppression of cortisol in plasma and saliva was calculated as (day 1 sample − day 2 sample)/day 1 sample (%).

Results

The subjects were aged 24–54 (mean 35·1) years with body mass indices ranging from 17·9 to 31·3 (means 27·1 in men *vs.* 23·5 in women, $P = 0{\cdot}001$ kg/m^2 and waist to hip ratios ranging from 0·82 to 1·2 (mean 0·94) in men and 0·67 to 0·81 (mean 0·74) in women. None of the subjects was depressed as indicated by the General Health Questionnaire. Table 1 shows the baseline and post-DXM suppression concentrations of

Table 1 Mean (95% confidence interval) fasting plasma cortisol and salivary cortisol concentration before and after suppression with 0·25 mg dexamethasone in duplicate tests carried out in 29 subjects at least one week apart

	Baseline		First test		Second test	
	Mean (95% CI)	Range	Mean (95% CI)	Range	Mean (95% CI)	Range
Plasma cortisol (nmol/l)	407.5 (362.27, 458.33)	232–958	270.4 (231.18, 316.33)	116–802	265.1 (226.60, 310.07)	105–853
Salivary cortisol (nmol/l)	15.3 (13.11, 17.94)	4.4–34.4	8.6 (6.65, 110.08)	1.6–29.2	7.3 (5.45, 9.82)	1.6–23.8

cortisol measured in plasma and saliva. The basal plasma cortisol concentrations ranged from 232 to 958 nmol/l (mean 407·5 nmol/l) and tended to be lower in the men than the women (371·4 *vs.* 485·6 nmol/l, $P=0{\cdot}06$). The mean concentration of cortisol measured after DXM suppression was similar for both tests as assessed by plasma (270·4 *vs.* 265·1 nmol/l) or saliva measurements (cortisol 8·6 *vs.* 7·3 nmol/l, total cortisol + cortisone, 37·6 *vs.* 29·4 nmol/l). Also the fractional suppression of plasma cortisol, which was similar on the two occasions (30·6% and 30·4%), was somewhat but not significantly greater than the fractional cortisol suppression measured in saliva (27·3% and 28·8%). Neither the fractional suppression nor the post-DXM cortisol measurements differed according to sex, age, body mass index or waist to hip ratio.

The reproducibility of the post-DXM cortisol concentrations obtained from the two tests is illustrated by the Bland–Altman plots in Figs 1 and 2 (showing the differences in cortisol concentration on the two occasions plotted against the mean). For plasma (Fig 1), the mean difference did not differ significantly from zero (mean 0·02, $P=0{\cdot}80$); 95% of differences were within 0·78 $\log_e$ units from the mean (−0·76–+0·80) indicating that a repeated measurement was likely to be approximately half as small or twice as large as the first. For saliva (Fig 2), the mean also did not differ significantly from zero (mean 0·16, $P=0{\cdot}29$) but 95% of differences were within 1·64 $\log_e$ units from the mean (−1·48–+1·80), thus indicating that a repeated measurement was likely to be approximately five times as small or five times as large as the first. Saliva repeatability was no better when cortisone was considered, either alone, when as a total of salivary cortisol and cortisone, or as a ratio of measurements.

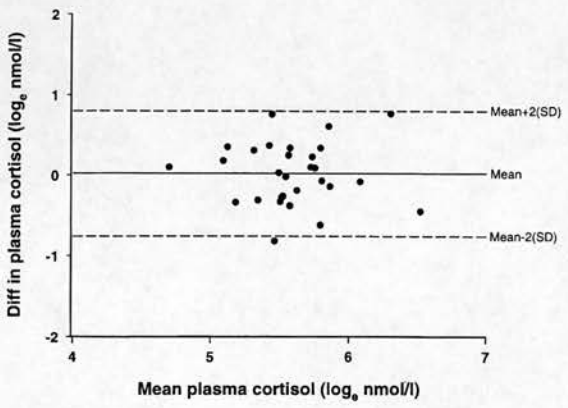

Fig. 1 Differences in $\log_e$ (post-DXM plasma cortisol) concentrations plotted against mean $\log_e$ concentration, $n=29$.

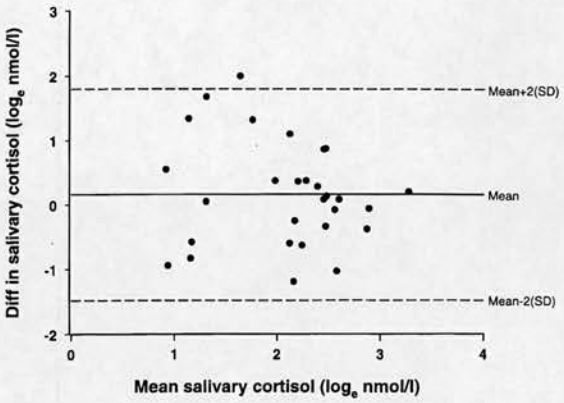

Fig. 2 Differences in $\log_e$ (post-DXM salivary cortisol) concentrations plotted against mean $\log_e$ concentration, $n=29$.

Discussion

This study suggests that the use of salivary cortisol measurement in the analysis of a very low dose overnight DST is less reproducible than plasma cortisol measurement. For saliva the repeated measurement was as much as five times as small or five times as large as the first for 95% of the time, while for plasma a repeated measurement was only half as small or twice as large as the first. Although the DST has gained increasing importance in studies examining the role of abnormalities of the HPAA in pathogenesis of several common diseases, there is surprisingly little information on the reproducibility of the test. Charles *et al.* (1982) showed similar levels of HPA axis suppression in 17 of 19 depressed patients studied on two occasions after the administration of 1 mg DXM. A similar conclusion was reached by Greden *et al.* (1983), who showed that nine of 13 subjects studied on two occasions had the same abnormal response to 1 mg DXM, defined by a threshold level of plasma cortisol suppression. However, in these studies of subjects with clear evidence of disease, no data was given on the reproducibility of the test within individuals. Importantly, the use of such a high dose of DXM would be expected to completely suppress the axis and hence be more likely to result in reproducibly undetectable cortisol levels. This contrasts with our study, where post-DXM cortisol levels were well within the detection limits of the assay, showing an approximately 30% suppression and hence a greater within-subject variability. The poorer performance of the 0·25 mg DST based on salivary measurements is difficult to explain. It was not due to assay imprecision, as the 'DELFIA' assay used in our studies has been shown to be reproducible over a wide range of cortisol concentrations and the assays for saliva and plasma had similar CVs. In addition, previous studies have shown that saliva

quantitatively reflects free plasma cortisol, and therefore might be expected to be a better index of DXM suppression. Also, although salivary cortisol is converted to cortisone by 11β-hydroxysteroid dehydrogenase Type 2 (Walker & Best, 1995) the salivary results were not significantly improved when the total of cortisol and cortisone concentrations were considered. Thus the reason for the poor reproducibility of the saliva measurement is unclear, but may include sampling problems (e.g. variable salivary flow rates) or a poor correlation between salivary and plasma cortisol concentrations at low (post-DXM) concentrations.

Our data imply that the intrinsic variation in salivary cortisol levels has been underestimated. The poorer repeatability of measurements compared to plasma suggests that in the context of field studies of dexamethasone suppression in normal individuals, salivary cortisol measurements may only be appropriate for large numbers of subjects.

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Altered Control of Cortisol Secretion in Adult Men with Low Birth Weight and Cardiovascular Risk Factors*

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ABSTRACT

It has been suggested that increased activity of the hypothalamic-pituitary-adrenal axis may link low birth weight with subsequent development of cardiovascular risk factors and disease. Two hundred and five men, aged 66–77 yr, who were born and still live in East Hertfordshire underwent an overnight very low dose (0.25 mg) dexamethasone suppression test followed by a low dose 1-μg ACTH-(1–24) stimulation test. A 24-h urine sample was collected for analysis of cortisol metabolites by gas chromatography/electron impact mass spectrometry. Men with lower birth weight had enhanced responses of plasma cortisol to ACTH-(1–24) ($P = 0.03$), increased total urinary cortisol metabolite excretion (after adjustment for confounding effects of increased obesity and lean body mass in high birth weight men; $P = 0.04$), but no difference in plasma cortisol after dexamethasone. Features of the metabolic syndrome were independently associated with enhanced adrenal responsiveness to ACTH-(1–24) (raised blood pressure, $P = 0.02$; glucose intolerance, $P = 0.09$; hypertriglyceridemia, $P = 0.02$), with trends to increased urinary cortisol metabolite excretion, but not with differences in plasma cortisol after dexamethasone. Men with low birth weight and/or the metabolic syndrome have increased activity of the hypothalamic-pituitary-adrenal axis. This may be an important mechanism underpinning the effects of events in early life on later cardiovascular disease. (*J Clin Endocrinol Metab* **86:** 245–250, 2001)

THE MECHANISMS underlying the association of type 2 diabetes, raised blood pressure, and dyslipidemia (*i.e.* the metabolic or insulin resistance syndrome) and their exacerbation by obesity are not known, but are important in our understanding of the pathophysiology of atheromatous disease. As patients with Cushing's syndrome develop these clinical features, it is an attractive idea that less profound disturbances of the hypothalamic-pituitary-adrenal axis (HPAA) might underlie the metabolic syndrome and its link with obesity. This idea is supported by case-control and cross-sectional studies that show that high blood pressure, glucose intolerance, insulin resistance, and hyperlipidemia are associated with elevated cortisol concentrations in blood, saliva, and urine (1–6) or impaired peripheral inactivation of cortisol (7). Increased secretion of cortisol also occurs in obesity, particularly if the obesity has a central distribution (8, 9). In addition, increased cortisol secretion could explain the link between psychosocial deprivation and cardiovascular risk (10).

Recent observational studies have shown that low birth weight predicts subsequent hypertension, insulin resistance, glucose intolerance, and cardiovascular disease (11). Events in early life may have long-term effects on the HPAA. Exposure of pregnant rats to adverse influences during gestation, including undernutrition, treatment with dexamethasone, alcohol, physical restraint, or nonabortive maternal infections, results in the birth of small offspring with hypertension and insulin resistance. These animals also have elevated basal or stress-induced glucocorticoid secretion (12–15). In studies of adult men, we recently reported that higher fasting morning plasma cortisol concentrations, a crude measure of cortisol secretion, are associated with higher blood pressure, plasma glucose and triglyceride concentrations, and lower birth weight (16, 17). Children and adolescents with lower birth weight have also been reported to excrete more cortisol or its metabolites in urine (18, 19). These studies have led to the hypothesis that events in early life permanently alter or program cortisol secretion, and that this together with increased obesity leads to a high prevalence of the metabolic syndrome and cardiovascular disease in adult life.

We investigated a group of men of known birth weight to test this hypothesis by characterizing abnormalities of cortisol secretion in relation to features of the metabolic syndrome, obesity, and birth weight.

Subjects and Methods

Subject selection

We previously studied a cohort of 370 men born between 1920 and 1930 in East Hertfordshire, UK, for whom birth weights were recorded by midwives. In 1991, blood pressure was measured, and 75-g oral glucose tolerance tests were performed (20). In 1997 we approached the surviving 245 men, of whom 205 were suitable and agreed to take part

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in the present study. Subjects with clinical evidence of pituitary or adrenal disease and those who had received oral glucocorticoids in the previous 3 months were excluded. Subjects were invited to attend on any convenient day between July and December. Ethical committee approval and written informed consent were obtained.

Clinical protocol

At a preliminary interview, information about medical and social history, family history of diabetes and hypertension, smoking habits, alcohol consumption, and current medication was recorded, and subjects completed a 10-item General Health Questionnaire to measure mood (21). On another occasion, subjects ingested 0.25 mg dexamethasone at 2200 h and fasted overnight. The following morning they attended a local clinic at 0830 h, a 21-gauge butterfly cannula was inserted in an antecubital vein, and, after 30 min rest, a baseline blood sample was obtained before 1.0 μg freshly diluted ACTH-(1–24) (tetracosactrin, Synacthen, Alliance, Chippenham, UK) was injected as a bolus with a 10 mL saline flush. Venous blood was sampled through the cannula at 20, 30, 40, and 60 min after ACTH-(1–24) administration. Samples were centrifuged immediately, and plasma was stored at −80 C. Height and weight were recorded, and waist and hip circumferences were measured with steel tape at the level of the umbilicus and greater trochanter, respectively. Finally, subjects collected a 24-h urine sample at least a week before or a week after the dexamethasone/ACTH-(1–24) test.

Dexamethasone (0.25 mg) and ACTH-(1–24) (1 μg) doses were selected to provide an average 50–75% maximal suppression or stimulation, respectively, with a wide range (22, 23). More conventional doses [*e.g.* 1 mg dexamethasone or 250 μg ACTH-(1–24)] would be expected to produce maximal effects in all of these otherwise healthy participants and would not allow detection of subtle alterations in the control of cortisol secretion.

Laboratory methods

Measurements of glucose, triglyceride, and insulin concentrations have been reported previously (20). RIAs were used to measure plasma cortisol with Guildhay antisera (24): corticosteroid-binding globulin (Medgenics Diagnostics, Fleurus, Belgium), dehydroepiandrosterone (Diagnostic Systems Laboratories, Inc., Webster, TX), 17α-hydroxyprogesterone (in-house RIA), progesterone (Immulite analyzer, Diagnostic Products Ltd., Gwynedd, Wales), and dexamethasone (enzyme-linked immunosorbent assay adapted from Cozart Biosciences Ltd., Abingdon, UK). Urinary creatinine was measured using the Jaffe reaction on the Bayer PLC (Newbury, UK) Advia analyzer.

Cortisol, cortisone, and their metabolites were measured in urine by gas chromatography/electron impact mass spectrometry (22, 25). Total cortisol metabolite excretion was calculated as tetrahydrocortisols (THFs) plus tetrahydrocortisone (THE) plus cortols plus cortolones. Ratios of urinary metabolites were used to infer relative activation of the principle enzymes metabolizing cortisol. Relative reduction by 5α- and 5β-reductases was inferred from the 5β-THF/5α-THF ratio. Whole body equilibrium between cortisol and cortisone, determined by the balance of tissue-specific activities of 11β-reductase and 11β-dehydrogenase activities, was inferred from the ratio of THFs/THE. Renal 11β-dehydrogenase activity was inferred from the urinary cortisol/cortisone ratio.

Statistical analysis

To obtain normally distributed variables, measurements of glucose, triglycerides, urinary cortisol metabolites, peak cortisol after ACTH-(1–24), and area under the curve after ACTH-(1–24) administration for dehydroepiandrosterone and 17α-hydroxyprogesterone, were $\log_e$ transformed. Geometric means and SDs are therefore presented for these variables. Associations between continuously distributed variables were assessed by the Pearson correlation coefficient, and associations between continuous and categorical variables were assessed by the Mann-Whitney U test or the two-sample *t* test as appropriate. Multiple linear regression was then used to explore the relationship between continuously distributed response variables and possible explanatory variables, with adjustment for confounding factors. Multiple logistic regression was used to analyze binary response variables. In addition to analyzing the peak cortisol response to ACTH-(1–24) using the methods described above, a longitudinal analysis of the cortisol response to ACTH-(1–24) was also conducted. The longitudinal approach considers the full series of cortisol data for each subject and models the average response during the test in relation to the factors of interest, taking into account the effects of time and the autocorrelation of cortisol measurements within each subject (26). All statistical analysis was carried out using STATA, release 5; the xtgee feature was used to implement the longitudinal analysis (Statacorp 1997, Stata Statistical software release 5, Stata Corp., College Station, TX).

Results

Subject characteristics and potential confounders

The men were aged between 66 and 77 (mean, 70.9; SD, 3.1) yr, with a mean body mass index (BMI) of 26.9 (SD, 3.7) kg/m^2. Fifteen men had type 2 diabetes (2 h glucose, ≥11.1 mmol/L), and 33 had impaired glucose tolerance (IGT; 2-h glucose, 7.8–11.0 mmol/L). Mean systolic blood pressure was 161.5 mm Hg (SD, 22.1), and 73 men were receiving treatment with antihypertensive drugs. Two subjects were excluded from analysis of plasma cortisol concentrations because of extreme values; one had a vaso-vagal event after iv cannulation, and the other was receiving ethinylestradiol treatment. Six men had missing values for glucose measurements. None of the measurements of cortisol in plasma or urine correlated with age or differed in subjects receiving topical or inhaled corticosteroid therapy (n = 16). None of the differences in plasma cortisol described below were accounted for by variation in plasma corticosteroid-binding globulin or dexamethasone concentrations (data not shown).

Obesity, reflected in increased BMI, was associated with higher blood pressure (r = 0.18; P = 0.04), hypertriglyceridemia (r = 0.28; P = 0.0001), and glucose intolerance (r = 0.16; P = 0.02). Obesity was also associated with a linear increase in total urinary cortisol metabolite excretion (r = 0.19; P = 0.006), but did not predict plasma cortisol after dexamethasone or ACTH-(1–24) administration. Central obesity, reflected in increased waist/hip ratio (WHR), was associated with similar trends. In addition, increased WHR predicted marginally lower plasma cortisol after 0.25 mg dexamethasone (r = −0.13; P = 0.06) and disproportionately higher excretion of 5α- rather than 5β-reduced metabolites of cortisol (r = −0.14; P = 0.05). Obesity was not associated with altered ratios of cortisol/cortisone metabolites. Increased lean body mass, as judged by urinary creatinine excretion (27), was also associated with higher total urinary cortisol metabolites (r = 0.23; P = 0.001). Neither obesity nor urinary creatinine was an independent predictor of total urinary metabolite excretion in multiple regression analysis. Urinary creatinine was not associated with plasma cortisol concentration.

Men with a current or previous history of depression (n = 12) had greater peak plasma cortisol concentrations after ACTH-(1–24) [474.0 (SD, 1.1) *vs.* 428.7 (SD, 1.2) nmol/L; P = 0.03] and higher total urinary cortisol metabolites [median, 22.4 (interquartile range, 16.3–48.2) *vs.* 17.8 (11.3–24.8) mg/24 h; P = 0.04]. Men with manual occupations (class IIIM—V; n = 134) had no difference in plasma cortisol concentrations, but excreted less total cortisol metabolites than men with nonmanual occupations [class I—IIIN; n = 69; 15.9 (SD, 2.1) *vs.* 20.8 (SD, 1.8) mg/24 h; P = 0.008].

Associations with birth weight

Table 1 shows relationships between birth weight and cortisol and its metabolites. A lower birth weight was associated with a greater rise in plasma cortisol concentrations after ACTH-(1–24) administration and a later peak time (Fig. 1), but no difference in plasma cortisol after dexamethasone administration. The inverse relationship between birth weight and adrenal ACTH-(1–24) responsiveness remained after exclusion of men with IGT and type 2 diabetes and/or treated hypertension and was not confounded by obesity. Differences in adrenocortical responses to ACTH-(1–24) in a subgroup of men with contrasting birth weight [>9.5 lb (4.31 kg) or ≤6.5 lb (2.92 kg); n = 12 in each group] were further explored by measurement of other ACTH-dependent adrenal steroids in plasma. Men with lower birth weight also had higher levels of dehydroepiandrosterone [mean area under curve: birth weight, ≤6.5 lb, 6.5 (SD, 2.2) nmol/L·h; >9.5 lb, 4.9 (SD, 1.5) nmol/L·h], 17α-hydroxyprogesterone [birth weight, ≤6.5 lb, 9.7 (SD, 1.3) nmol/L·h; >9.5 lb, 8.1 (SD, 1.3) nmol/L·h], and progesterone [birth weight ≤6.5 lb, 3.6 (SD, 0.5) nmol/L·h; >9.5 lb, 3.1 (SD, 0.7) nmol/L·h], indicating that no common biosynthetic defect, such as 21-hydroxylase deficiency, accounts for the difference in cortisol response.

Total urinary cortisol metabolite excretion was higher in men with the lowest and highest birth weights ($P = 0.03$ for quadratic trend). Although men with higher birth weight were not more obese, they were taller and heavier as adults ($r = 0.23$; $P = 0.001$) and excreted more creatinine in urine ($r = 0.20$; $P = 0.006$), which could confound the relationship with urinary glucocorticoid excretion. After adjustment for urinary creatinine and obesity (either BMI or WHR), an inverse linear relationship, rather than a quadratic trend, was evident, such that low birth weight men excreted more urinary cortisol metabolites ($r = -0.29$; $P = 0.04$). Birth weight was not associated with cortisol metabolite ratios (data not shown).

Associations with features of the metabolic syndrome

Table 2 shows relationships of plasma cortisol and urinary cortisol metabolites to blood pressure, glucose tolerance, and fasting plasma triglyceride concentrations after correction for the potential confounding effects of obesity, depression, social class, and urinary creatinine where appropriate. Plasma cortisol concentration at 0900 h after dexamethasone treatment was not associated with cardiovascular risk factors. However, the peak plasma cortisol concentration after ACTH-(1–24) was higher in men with higher blood pressure and higher fasting plasma triglyceride concentrations and tended to be higher in men with higher post-glucose plasma glucose concentrations. Likewise, in a longitudinal analysis of the cortisol profiles, there were similar positive associations between mean plasma cortisol concentration and these features, with or without adjustment for potential confound-

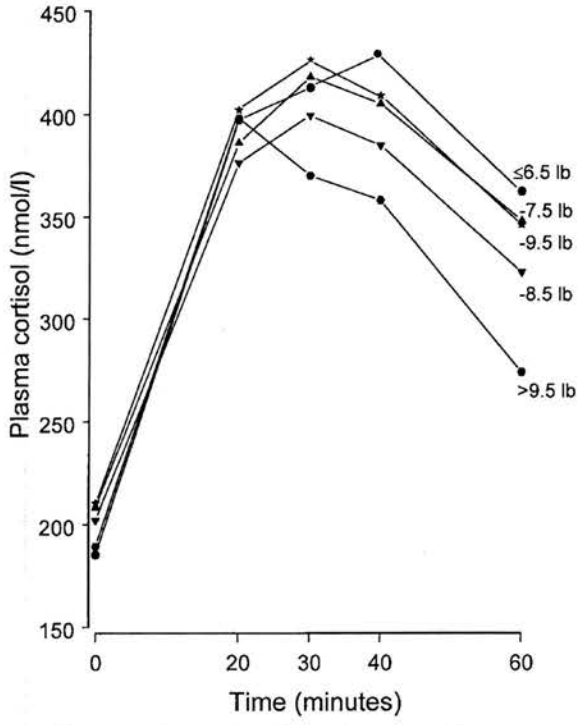

FIG. 1. Plasma cortisol profiles during short Synacthen test according to birth weight. $P = 0.03$ from longitudinal analysis, interaction of cortisol response by birth weight with time. ●, Birth weight of 6.5 lb (2.92 kg) or less; ▲, 7.5 lb (3.41 kg); ▼, 8.5 lb (3.86 kg); ★, 9.5 lb (4.31 kg); ●, >9.5 lb (4.31 kg).

TABLE 1. Relationships between birth weight and cortisol and its metabolites

Birth wt in pounds (kg)	No. of men	Post-dexamethasone plasma cortisol (nmol/L)[a]	Peak plasma cortisol (nmol/L)[b,c]	Total urinary cortisol metabolites (mg/24 h)[b]	Total urinary cortisol metabolites/creatinine excretion (mg/mmol)[b]
≤6.5lb (2.92)	26	185.3 (88.7)	458.2 (1.2)	22.8 (1.7)	7.24 (1.7)
−7.5lb (3.41)	59	208.2 (92.9)	436.1 (1.2)	15.4 (2.2)	3.74 (2.2)
−8.5lb (3.86)	73	202.0 (94.9)	417.0 (1.2)	17.3 (2.2)	4.10 (2.1)
−9.5lb (4.31)	33	210.2 (98.2)	444.5 (1.2)	14.1 (1.9)	3.35 (2.0)
>9.5lb (4.31)	12	189.3 (80.2)	405.3 (1.2)	23.6 (1.7)	5.26 (1.7)
All	203	202.2 (92.8)	431.3 (1.2)	17.1 (2.1)	4.22 (7.8)
		NS	0.03[c]		0.10[c]
			0.03[d]		0.04[d]
				0.03[e]	0.07[e]

[a] Arithmetic mean (SD) for 0900 h plasma cortisol after 0.25 mg dexamethasone.
[b] Peak plasma cortisol after 1 μg ACTH-(1–24) calculated from individual maximums rather than mean data in Fig. 1. Geometric mean (SD).
[c] Unadjusted P value.
[d] P value adjusted for BMI, depression, and social class.
[e] Unadjusted P value for quadratic trend.

TABLE 2. Relationships between features of the metabolic syndrome and cortisol and its metabolites

		No. of men	Plasma cortisol post-dexamethasone (nmol/L)[a]	Peak plasma cortisol post-ACTH-(1–24) (nmol/L)[b]	Cortisol profile	Total urinary cortisol metabolites (mg/24 h)[b]	Total urinary cortisol metabolites/creatinine (mg/mmol)[b]
Glucose tolerance	Normal IGT/ type 2 diabetes[c]	149	199.7 (91.0)	427.5 (1.2)		16.2 (2.0)	3.97 (1.97)
		48	213.2 (101.5)	442.3 (1.2)		19.7 (2.3)	5.00 (2.34)
P value			NS	0.09[d] 0.15	0.05[d] 0.06	NS	NS
Blood pressure	ý 160 mm Hg	83	204.8 (101.2)	415.7 (1.2)		17.1 (1.8)	4.22 (1.78)
	>160 mm Hg or treatment	120	200.5 (86.9)	442.2 (1.2)		17.0 (2.3)	4.17 (2.26)
P value			NS	0.02[d] 0.03[e]	NS	NS	NS
Triglycerides	ý 1.4 mmol/L	102	196.8 (90.7)	418.0 (1.2)		15.6 (2.1)	3.82 (2.09)
	>1.4 mmol/L	101	207.8 (95.0)	445.3 (1.2)		18.6 (2.0)	4.66 (1.54)
P value			NS	0.02[d] 0.04[e]	0.08[d] 0.06[e]	NS	NS
Metabolic syndrome[c]	Absent	166	197.2 (90.5)	424.8 (1.2)		16.7 (2.0)	4.14 (1.99)
	Present	31	234.3 (104.9)	466.4 (1.2)		18.4 (2.5)	4.53 (2.49)
P value			NS	0.01[d] 0.04[e]	0.007[d] 0.006[e]	NS	NS
All		203	202.2 (92.8)	431.3 (1.2)		17.1 (2.1)	4.2 (7.8)

[a] Arithmetic mean (SD).
[b] Geometric mean (SD).
[c] Defined in text.
[d] *P* value unadjusted.
[e] *P* value adjusted for BMI, social class, and depression.

ing factors. In men with all three features of the metabolic syndrome [previously defined (28) as systolic blood pressure >160 mm Hg or subject receiving antihypertensive therapy (n = 120), the presence of impaired glucose tolerance or type 2 diabetes (n = 48), or fasting plasma triglyceride >1.4 mmol/L; n = 101], peak and mean plasma cortisol concentration over time were significantly elevated. Total urinary cortisol metabolite excretion also tended to be greater in men with these cardiovascular risk factors.

Predictors of the metabolic syndrome

Having examined individual features of the metabolic syndrome, logistic regression modeling was then performed to identify predictors of combined features of the metabolic syndrome (as defined above). Potential variables included age, social class, birth weight, WHR, BMI, plasma cortisol after dexamethasone, peak plasma cortisol after ACTH-(1–24), total urinary cortisol metabolite excretion, and ratio of 5β-/5α-reduced metabolites of cortisol. The best fitting model identified effects of BMI ($P = 0.003$), peak plasma cortisol after ACTH-(1–24) ($P = 0.03$), and birth weight ($P = 0.02$). The effect of birth weight was more significant when peak plasma cortisol was excluded ($P = 0.008$), and the effect of peak plasma cortisol was more significant when birth weight was excluded ($P = 0.02$). The estimated odds ratios for the metabolic syndrome are 1.18 (95% confidence interval, 1.06–1.32) for a unit increase in BMI, 1.55 (95% confidence interval, 1.09–2.21) for a 1-lb decrease in birth weight, and 1.28 (95% confidence interval, 1.02–1.61) for a 50 nmol/L increase in peak cortisol concentration. The effects of WHR were similar to those of BMI.

Discussion

The hypothesis that cortisol contributes to the pathogenesis of essential hypertension and type 2 diabetes was rejected by many on the basis of small case-control studies with very limited measurements of cortisol secretion (29, 30). More recently, a series of cross-sectional studies identified associations between increased cortisol levels and higher blood pressure (1–4, 6), obesity (5, 8, 9), and lower birth weight (16–18). The current report describes the most detailed assessment to date of the regulation of cortisol secretion in a cross-sectional study of men in whom the prevalence of the metabolic syndrome and its antecedents, including obesity and birth weight, have been carefully characterized. The principal findings are that men with low birth weight and features of the metabolic syndrome have enhanced responsiveness of plasma cortisol to ACTH-(1–24) and increased total urinary cortisol metabolite excretion, but normal plasma cortisol after dexamethasone. Indeed, plasma cortisol after ACTH-(1–24) accounted for much of the effect of birth weight on features of the metabolic syndrome. These findings are consistent with the hypothesis that low birth weight is associated with increased activity of the HPAA and that this could contribute to the metabolic syndrome and the attendant risk of cardiovascular disease.

The explanation for activation of the HPAA in men with lower birth weight and features of the adult metabolic syndrome remains unclear. Rats exposed to glucocorticoids *in utero* have increased plasma glucocorticoid levels, which have been associated with lower levels of glucocorticoid receptors in brain and pituitary gland, which may impair negative feedback control of CRH and ACTH secretion (14).

If the same programming of glucocorticoid receptor expression occurred in man, then suppression of plasma cortisol by dexamethasone would be expected to be impaired in men with lower birth weight, but we found that it was preserved. Indeed, as fasting plasma cortisol was higher in low birth weight men without dexamethasone (16, 17), but was not different after dexamethasone administration, the incremental effect of dexamethasone may be greater. However, dexamethasone may not cross the blood-brain barrier adequately at low doses in man (31), so this only tests the pituitary component of the negative feedback loop. Alternatively, elevated plasma cortisol may result from enhanced drive to CRH, ACTH, and cortisol secretion from higher centers manifest as an increase in plasma cortisol when stressed on first sampling. Or increased cortisol secretion could result from increased adrenocortical sensitivity to ACTH. Our measurements of other ACTH-dependent steroids exclude variance in cortisol response due to subclinical 21-hydroxylase deficiency (32). Other corticosteroid biosynthetic defects that have been proposed as being important in hypertension, such as 11β-hydroxylase deficiency, predict lower, rather than higher, cortisol responses. Finally, the pattern of cortisol response to Synacthen in low birth weight subjects with both an increased peak and a slower decline suggests that they may have impaired plasma clearance of cortisol not revealed by 24-h urinary cortisol analysis.

Any of these possible mechanisms of altered cortisol secretion could be subject to programming by events in early life. Alternatively, there may be genetic determinants underlying cortisol secretion that also impact on fetal development. There are relationships between patterns of cortisol secretion and metabolism within families (33), and increased cortisol secretion has been shown to be inherited together with higher blood pressure (4). As birth weight is also at least in part inherited with higher blood pressure (34), and as increased glucocorticoid exposure *in utero* can lead to low birth weight (13), a genetic alteration in cortisol secretion could explain associations between birth weight and subsequent hypertension without the need to invoke programming.

By contrast with cortisol measurements in low birth weight men, our data show that obesity is associated with lower plasma cortisol after dexamethasone treatment and no difference in responses to ACTH-(1–24) in the face of increased urinary cortisol metabolite excretion, especially of 5α-reduced metabolites of cortisol. The lower plasma cortisol may be explained by increased peripheral metabolism of cortisol by 5α-reductases (35). Indeed, increasing obesity and its associated increase in lean body mass (reflected in higher creatinine excretion) among high birth weight men confounded the relationship between birth weight and urinary cortisol metabolite excretion. This confounding effect resulted in a U-shaped unadjusted relationship between birth weight and cortisol metabolites, as described previously (18). Although obesity may amplify the metabolic syndrome and its association with low birth weight (36), these data suggest that primary changes in cortisol in the lean insulin resistance syndrome are not the same as those in primary obesity.

In conclusion, these data suggest that men with the cluster of cardiovascular risk factors that includes low birth weight and the adult metabolic syndrome have activation of the HPAA. This may be a key mechanism to explain the relationship between low birth weight and subsequent cardiovascular disease and may offer novel therapeutic strategies to reduce cardiovascular risk.

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Elevated Plasma Cortisol in Glucose-Intolerant Men: Differences in Responses to Glucose and Habituation to Venepuncture

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ABSTRACT

Recent evidence suggests that variations in cortisol activity within the physiological range contribute to associations between multiple cardiovascular risk factors. Plasma cortisol measurements during a glucose tolerance test differ in men with hypertension, insulin resistance, and glucose intolerance, but it is unclear whether this reflects altered responses of cortisol to glucose, altered circadian rhythm, or altered habituation to multiple sampling. We performed a single-blind randomized cross-over study comparing 75 g oral glucose with placebo in 39 fasted men (22 glucose intolerant and 17 controls) aged 68–77 yr. In all subjects, plasma cortisol fell during the glucose tolerance test. Subjects with glucose intolerance had significantly higher plasma cortisol following placebo ($P = 0.001$), suggesting an altered circadian rhythm. Treatment with an oral glucose load blunted the circadian fall in plasma cortisol ($P = 0.002$), but this response was no different in controls or glucose intolerant subjects. In addition, 0900 h plasma cortisol was higher in the first study phase in controls ($P = 0.01$) but not in glucose-intolerant subjects ($P = 0.18$), who showed a lack of habituation to repeated plasma measurements. These data support the hypothesis that alterations in central regulation of the hypothalamic-pituitary-adrenal axis may be important in glucose intolerance. (*J Clin Endocrinol Metab* **86:** 1149–1153, 2001)

PATIENTS with high circulating cortisol due to Cushing's syndrome develop glucose intolerance, insulin resistance, and other risk factors for cardiovascular disease including hypertension and dyslipidemia. There is now increasing evidence that physiological variations in cortisol action could also contribute to development of these risk factors. Plasma cortisol and the cortisol response to the administration of corticotrophin are increased in men and women with type 2 diabetes mellitus (1, 2). Furthermore, differences in activity of the hypothalamic-pituitary-adrenal (HPA) axis have been demonstrated in subjects with cardiovascular risk factors. These include elevated morning plasma cortisol (3–7), increased excretion of cortisol metabolites (7, 8), abnormalities of diurnal cortisol secretion (9), and impaired peripheral inactivation of cortisol (7).

The mechanisms leading to variations in plasma cortisol action in subjects with glucose intolerance are unknown. Differences in glucose and/or insulin concentrations may be important: for example, insulin may modulate adrenal steroidogenesis by inhibiting 17,20-lyase, thereby favoring cortisol synthesis in preference to dehydroepiandrosterone and androstenedione (10). Insulin may also affect cortisol metabolism by decreasing the activity of 11β-hydroxysteroid dehydrogenase type 1, which acts primarily as a reductase mediating conversion of inactive cortisone to active cortisol (11). Such effects may be important determinants of acute changes in plasma cortisol, for example during a glucose tolerance test. It is known that plasma cortisol levels fall during a glucose tolerance test (12, 13) and that this effect may differ in subjects with cardiovascular risk factors (14, 15). However, it is not known whether this reflects the circadian fall in circulating plasma cortisol and whether ingestion of glucose affects the response. We therefore performed a placebo-controlled study of the effect of oral glucose on circulating plasma cortisol. To further explore the associations between cortisol and glucose tolerance, we studied both subjects with glucose intolerance and normoglycaemic controls.

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Subjects and Methods

Participants

We studied 40 men, aged 68 to 77 yr, selected from a well-characterized cohort from Hertfordshire who have participated in previous investigations of the relationships between early life events and subsequent Type 2 diabetes (16). The subjects were selected by their previous glucose tolerance data from 1991 with the aim of studying equal numbers with glucose intolerance and normal controls. None had a history of endocrine disease or had received systemic or topical glucocorticoid treatment within the previous 6 months. Ethical approval was obtained

from East and North Hertfordshire Local Research Ethics Committee and written informed consent was obtained.

Clinical protocol

Following an overnight fast, subjects attended a local clinic at 0830 h for oral glucose tolerance tests. A 21-g butterfly cannula was inserted in an antecubital vein and after 30-min rest, a baseline blood sample was obtained. Subjects then drank either 75 g oral glucose (as 389 ml Traditional Lucozade Sparkling Glucose Drink) or placebo (identical in appearance and taste to Lucozade but containing no glucose, supplied by SmithKline Beecham). They returned a week later for a repeat test with the alternative solution in a single-blind cross-over design. Twenty-nine subjects (17 glucose intolerant, 12 controls) received glucose in the first phase, whereas 10 subjects (5 glucose intolerant, 5 controls) received placebo first. Venous blood was sampled from the cannula at 30, 60, 90, and 120 min following the glucose or placebo load. Placebo and glucose phases were separated by at least 1 week.

Laboratory methods

Blood samples were centrifuged, processed immediately and stored at −80 C for subsequent hormone analysis. RIAs were used to measure plasma cortisol using Guildhay antisera (17) and corticosteroid-binding globulin (CBG) (Medgenics Diagnostics, Fleurus, Belgium). Plasma glucose was measured by the hexokinase method.

Statistical methods

As the distributions of cortisol measurements were skewed, $\log_e$ transformed variables were used in all analyses. Independent two-sample t tests were used to compare cortisol concentrations for control compared with glucose intolerant subjects. ANOVA for repeated measures was used to analyze the plasma cortisol measurements during the glucose tolerance test.

Results

One subject completed only one phase of the study and was therefore excluded from analysis. Using plasma glucose measurements following the 75 g oral glucose load, we defined 22 subjects as glucose intolerant [either impaired glucose tolerance (2 h plasma glucose 7.8 to 11.0 mmol/L) or Type 2 diabetes (2 h plasma glucose ≥ 11.1 mmol/L)], and 17 subjects as normal controls.

Differences in plasma cortisol between controls and glucose intolerant subjects without glucose

In all subjects, plasma cortisol concentrations declined over the 120 min of the test following placebo. Glucose intolerant subjects had significantly higher cortisol concentrations following placebo than controls ($P = 0.001$) (Fig. 1). This difference was most marked at baseline and during the first 90 min of the test but was no longer present at 120 min. The differences in cortisol between glucose intolerant subjects and controls were not accounted for by variations in CBG (data not shown).

Effect of treatment with glucose on plasma cortisol

Figure 2 shows that treatment with an oral glucose load blunted the circadian fall in plasma cortisol in both controls and glucose intolerant subjects ($P = 0.002$). There was no significant difference in the effect of the glucose load in controls compared with those with glucose intolerance (controls $P = 0.02$, glucose intolerant $P = 0.04$, interaction $P = 0.50$).

Order of test: habituation to venepuncture

0900 h plasma cortisol concentrations were significantly higher in the first phase of the study than in the second phase in controls ($P = 0.01$) (Fig. 3). However, in glucose intolerant subjects, 0900 h plasma cortisol concentrations in the two-study phases were not different ($P = 0.18$), as this measurement did not fall in the second study phase.

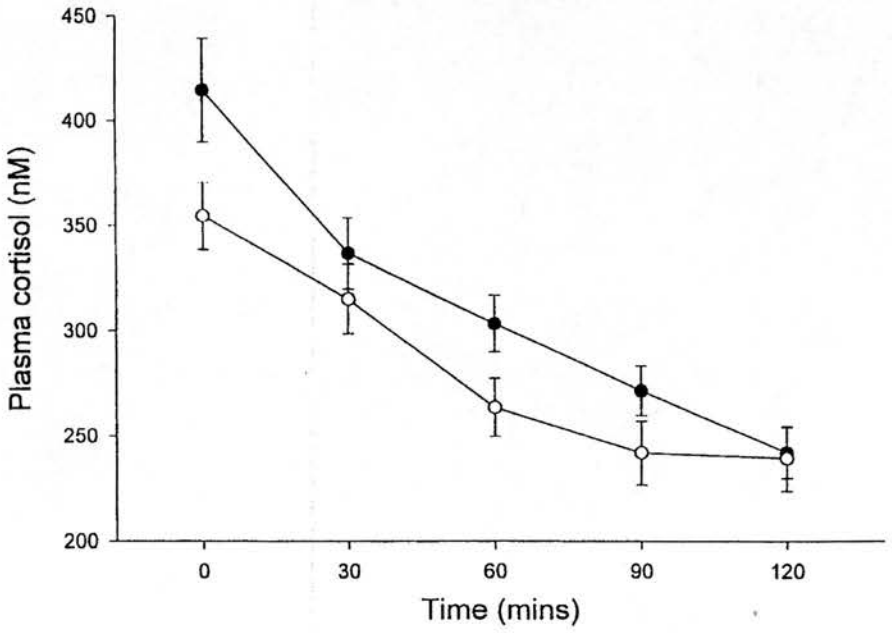

FIG. 1. Differences in plasma cortisol between controls and glucose intolerant subjects following placebo. $P = 0.001$ from ANOVA for repeated measures for difference in plasma cortisol following placebo between glucose intolerant (–●–) and control (–○–) subjects.

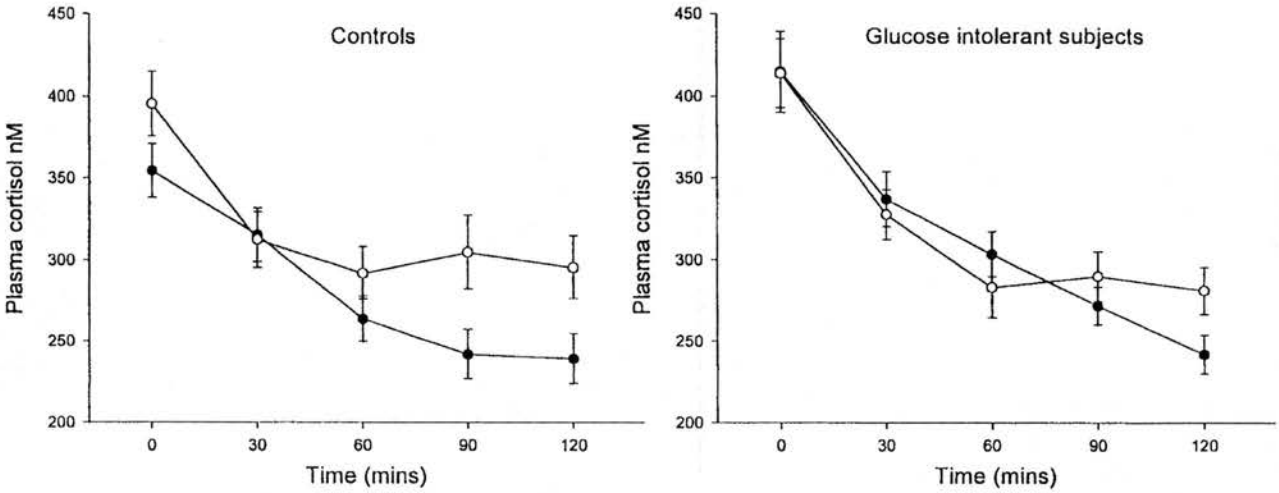

FIG. 2. Effect of glucose on plasma cortisol. –●– plasma cortisol following placebo. –○– plasma cortisol following glucose. $P = 0.002$ from ANOVA for repeated measures for effect of glucose in raising plasma cortisol in all subjects. No significant difference in effect of glucose between controls and glucose intolerant subjects ($P = 0.02$ for controls, $P = 0.04$ for glucose intolerant subjects).

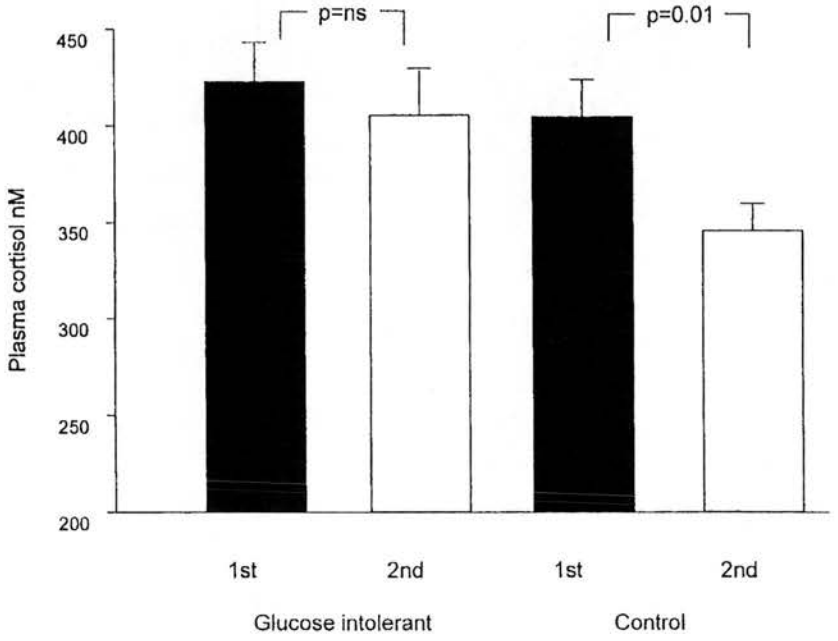

FIG. 3. Effect of order on 0900 h plasma cortisol effect of order of study phase (first phase, *black columns*, second phase, *white columns*) on 0900 h plasma cortisol in glucose intolerant and control subjects. $P = 0.01$ for effect of order in control subjects; no significant order effect in glucose intolerant subjects.

Effect of treatment with glucose and order of test on plasma glucose and insulin concentrations

Both plasma glucose and insulin concentrations were higher after treatment with glucose than placebo ($P < 0.001$) and were significantly higher in glucose intolerant subjects than controls. In contrast to the plasma cortisol concentrations, plasma glucose and insulin concentrations were no different in the two study phases in either glucose intolerant subjects or controls.

Discussion

We have shown that treatment with an oral glucose load blunts the normal circadian fall in plasma cortisol in subjects with glucose intolerance and in normoglycaemic controls. However, subjects with glucose intolerance had higher cortisol concentrations following placebo, suggesting that their normal diurnal fall in plasma cortisol differed from controls. We also found that baseline plasma cortisol concentrations were higher during the first phase of the study in control subjects suggesting a stress response associated with the novelty of first clinic attendance. In addition, subjects with glucose intolerance had high plasma cortisol concentrations at the second clinic attendance suggesting a lack of habituation to repeated measurement of plasma cortisol.

Our findings accord with early reports showing that plasma cortisol concentrations fall during an oral glucose

tolerance test (12, 13). It was not known whether this reflects the fall in circadian fall in plasma cortisol or whether glucose ingestion affects the response. We performed our studies in the morning when any alterations in circadian rhythm should be most clearly demonstrated. Indeed, we found higher plasma cortisol in subjects with glucose intolerance than controls during the first 90 min of the placebo phase of the study. Altered diurnal rhythms of salivary cortisol in subjects with glucose intolerance have been reported (9), and our finding is consistent with the previously reported associations between 0900 h plasma cortisol and glucose intolerance (5, 6). However, the difference between subjects with glucose intolerance and controls was no longer apparent at 120 min. This finding could explain why associations between cortisol measurements and glucose tolerance are still evident at 120 min following glucose in some reports (14) but to a lesser extent than the fasting measurements in others (15). The current study, therefore, implies that where two measurements of plasma cortisol are separated by 2 h, during which cortisol will fall due to diurnal variation, the timing of measurements is critical.

Despite the differences in circadian fall of plasma cortisol, the effect of glucose ingestion was to raise plasma cortisol in both normal and glucose intolerant subjects. Plasma and salivary cortisol concentrations rise following a meal (9), particularly if the meal is of high protein content (18), as protein ingestion stimulates pituitary ACTH secretion (19). Although it has previously been suggested that carbohydrate ingestion has little effect on the hypothalamic-pituitary-adrenal (HPA) axis (20), we have now demonstrated that an oral glucose load raises plasma cortisol. It remains unknown whether this is a consequence of the glucose load itself, or whether the associated insulin release affects cortisol metabolism (10, 11). Insulin may also act centrally on the HPA axis regulating drive, although results of studies to date are conflicting, either showing a decreased cortisol response to CRH following insulin infusion (21), or increased adrenocorticotrophic hormone (ACTH) secretion following supraphysiological hyperinsulinaemia (22). Likewise, the changes in cortisol induced by the glucose load could influence subsequent glucose and insulin metabolism. Manipulation of cortisol levels within the physiological range alters insulin sensitivity (23). And although subject to the limitations of measurement of hepatic glucose output in man, cortisol has been shown to impair insulin-dependent glucose uptake in the periphery and enhance gluconeogenesis in the liver (24, 25). However, as the effect of treatment with the glucose load did not differ between glucose intolerant subjects and controls, it would appear less likely that the hyperglycaemia or changes in insulin concentrations associated with glucose intolerance influenced the fall in cortisol concentrations during the test.

One alternative hypothesis to explain the elevated plasma cortisol during the glucose tolerance test is a response to stress. We attempted to reduce any effect of stress by conducting the study in familiar surroundings, with staff previously known to the subjects. Yet we have found that fasting plasma cortisol measured at the first phase attendance, and so arguably the most stressful visit, was significantly higher than that at the second phase in control subjects. Elevated plasma and salivary cortisol concentrations are observed in situations of increased perceived stress (9), and the stress of venepuncture is known to raise plasma cortisol (26). The effect of psychosocial stress on raising cortisol has also been reported to be greater after a glucose load (27).

Most interestingly, we found that glucose intolerant subjects also had high baseline plasma cortisol concentrations in the second phase of the study, indicating a lack of habituation to repeated stress. A similar lack of habituation of blood pressure and heart rate responses to repeated restraint stress is seen in spontaneously hypertensive rats compared with normal controls (28). Subjects with glucose intolerance have evidence of increased activation of the HPA axis (29), and so elevated plasma cortisol from stress of venous sampling would be consistent with enhanced drive to CRH, ACTH, and cortisol secretion from higher centers in these subjects. Lack of habituation to stress and increased activation of the HPA axis in subjects with glucose intolerance would support the hypothesis that chronic stress in man leads to development of cardiovascular risk factors. Such variations in HPA axis activity may also contribute to the observed relationships between psychosocial stress and subsequent cardiovascular disease (30).

In conclusion, this study supports the hypothesis that alterations in central regulation of the HPA axis may be an important mechanism underlying the development of glucose intolerance and subsequent cardiovascular disease.

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tions did have a significantly longer duration of diabetes (14.2 vs 6.4 years; $P<.001$).

Of the 17 patients with CHF, 4 patients had a documented New York Heart Association functional classification (class II: n=2; class III: n=2). Of the 8 patients with renal dysfunction, the mean serum creatinine was 1.8 mg/dL (159.1 μmol/L) and mean blood urea nitrogen was 27 mg/dL (9.639 mmol/L). Only 2 patients had documentation in the medical record that providers considered metformin contraindications.

Comment. In our review, almost one quarter of patients with a prescription for metformin had 1 or more absolute contraindications. Several recent studies in Europe have documented similar rates of inappropriate metformin prescribing.[2,5,6]

Adverse event reports suggest the incidence of metformin-associated lactic acidosis is between 1 in 10 000 to 1 in 100 000 patient-years.[7] In the first 14 months after its release in the United States, the FDA received 47 confirmed cases of lactic acidosis associated with metformin, with a 42% mortality rate. More than 90% of patients had relative or absolute contraindications to metformin.[3]

Because our assessment of the prevalence of contraindications to metformin use relies on a chart review, it may underestimate the frequency of contraindications and it is difficult to determine whether clinicians are aware they are prescribing metformin against a black-box warning. Nonetheless, our results suggest that metformin frequently may be inappropriately prescribed despite black-box contraindications. Documentation of this potential risk in the medical record is limited and health care providers should consider improving the documentation of the risk of lactic acidosis and provide appropriate counseling for patients who receive the drug.

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Skeletal Muscle Glucocorticoid Receptor Density and Insulin Resistance

To the Editor: In Cushing syndrome, elevated plasma cortisol levels cause insulin resistance, hyperglycemia, hypertension, and dyslipidemia. In patients without Cushing syndrome, these cardiovascular risk factors are associated with more subtle elevations in plasma cortisol concentrations[1] and enhanced tissue responsiveness to glucocorticoids.[2] We explored the possibility that insulin resistance in patients without Cushing syndrome involves dysregulation of glucocorticoid receptor (GR) expression in muscle.

Methods. We obtained biopsies of vastus lateralis skeletal muscle under local anesthesia from 23 men without fasting hyperglycemia participating in the the Uppsala Longitudinal Study of Adult Men.[3] As previously described, participants underwent a 75-g oral glucose tolerance test, euglycemic hyperinsulinemic clamp, and ambulatory blood pressure recording. Height, weight, and waist and hip circumferences were measured. Glucocorticoid receptor messenger RNA (mRNA) levels were measured in muscle total RNA using a quantitative reverse transcriptase (RT) polymerase chain reaction (PCR) assay with synthetic RNA competitors for GR mRNA and 18S mRNA as internal control. Both competitors contained 83 base pair deletions to distinguish PCR products derived from endogenous and synthetic RNAs.[4] The interassay coefficient of variation was 12%. Stata v5.0 (Stata Corp, College Station, Tex) was used for all analyses.

Results. Results are shown in the TABLE. After adjusting for body mass index (BMI), higher levels of skeletal muscle GR mRNA were associated with hypertension, higher insulin levels after a glucose load, and insulin resistance in a euglycemic hyperinsulinemic clamp. Muscle GR mRNA was not associated with plasma lipids, glucose, or BMI alone.

Comment. These data show that men with insulin resistance and hypertension have increased GR mRNA levels and, by inference, increased numbers of GRs in skeletal muscle. Glucocorticoid receptors mediate diverse effects on insulin sensitivity (in liver, adipose tissue, and skeletal muscle) and blood pressure (in kidney, blood vessels, and brain). Increased numbers of receptors in these sites could contribute to the association between features of the insulin resistance syndrome, and explain enhanced responsiveness to glucocorticoids.[2] Glucocorticoid receptors also contribute to negative feedback regulation of the hypothalamic-pituitary-adrenal axis. If receptor expression were similarly increased in central feedback sites, it might be expected that lower circulating cortisol levels would compensate for peripheral hypersensitivity.

Table. Associations of GR mRNA Levels With Cardiovascular Risk Factors*

Risk Factor	Mean (SD)	Partial Correlation Coefficient†	*P* Value	*P* Value Adjusted for BMI
Fasting plasma glucose, mg/dL‡§	99.1 (16.2)	−.05	.80	.58
Fasting plasma insulin, μIU/mL‡§	1.5 (1.2)	0.41	.06	.09
Insulin 1 h postglucose, μIU/mL‡§	10.7 (6.8)	.53	.01	.02
Insulin sensitivity as M/I (mg·min^{-1}·kg^{-1}[mU/1]$^{-1}$)‖	5.2 (2.0)	−.36	.10	.05
24-Hour systolic blood pressure, mm Hg¶	142	NA	.05	.05
Triglycerides, mg/dL‡§	106.2 (79.6)	0.26	.24	.30
BMI, kg/m^2	26.3 (3.3)	0.16	.46	Reference
Waist-hip ratio	0.95 (0.05)	0.14	.53	.91

*GR indicates glucocorticoid receptor; mRNA, messenger RNA; BMI, body mass index; and NA, not applicable.
†All associations with GRs are adjusted for 18s mRNA.
‡Median and interquartile range are presented, but variables were $\log_e$ transformed for statistical comparisons.
§To convert mean (SD) glucose values to mM, multiply by 0.0555; to convert insulin values to pM, multiply by 6.945; to convert triglyceride values to mM, multiply by 0.0113.
‖M/I = glucose infusion rate (g·min^{-1}) between 60 and 120 minutes of the euglycemic clamp divided by body weight (kg) and mean insulin level (mU·1^{-1}).
¶Associations with 24-hour mean ambulatory blood pressure were examined by censored normal regression to discount the effect of antihypertensive therapy. The 6 men receiving antihypertensive therapy and the 3 with untreated values above 150 mm Hg were allocated to the top tertile; median is presented and no partial correlation coefficient can be calculated.

However, dysregulation of GR expression appears to be tissue-specific. In an animal model of insulin resistance, dysregulation of GR expression was associated with increased GR mRNA in the liver but decreased GR mRNA in central negative feedback sites.[5] Further understanding of tissue-specific variations in GR expression and function may offer fundamental insights into the pathophysiology of insulin resistance and its association with hypertension.

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